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APPARATUS AND METHODS FOR MANIPULATING NUCLEAR MATERIAL

BACKGROUND OF THE INVENTION

[0001] THIS INVENTION relates to apparatus and methods for manipulating nuclear material. This invention has particular but not exclusive application to apparatus and methods for the isolation and purification of cell nuclei and nucleic acids such as genomic DNA and RNA from plant tissues and for illustrative purposes reference will be made to such an application. However, it is understood that this invention could be used for isolation and purification of cell nuclei and nucleic acids from the tissues of other genera and species, such as from tissues of animals (mammals, reptiles, birds, amphibia, fish, insects), fungi and bacteria.

[0002] That biochemical and molecular genetic studies of plants are hindered by the difficulties in the extraction of pure DNA and other nucleic acids, from many different tissues, has been long recognised. The difficulties encountered in isolating full length genomic DNA and undegraded RNA from plants may be the result of several factors. These factors may include: (A) the need to mechanically disrupt the robust cellulose wall of plant cells, which may lead to release of secondary products from storage vacuoles, or cause damage to cell organelles, and (B) because of the presence of a large variety and quantity of "plant secondary products", such as phenolic and polyphenolic compounds, polymeric carbohydrates and proteins, including abundant nucleases and phenol oxidases which can initiate processes that digest the DNA or cross-link the DNA, polysaccharides and proteins with aromatic polymeric compounds.

[0003] Disruption of plant tissues during DNA isolation causes release of abundant soluble polysaccharides from the plant cell walls, while tannins and phenolic compounds are released from the vacuole and intracellular stores. In the presence of oxygen, phenol oxidases may rapidly catalyse the polymerization of phenols, causing the characteristic browning seen in damaged plant tissues. The extensive cross-linking of these reactive

species with protein and other biopolymers may also occur. Many of these contaminating compounds are released following plant cell disruption and may be readily co-extracted with the nucleic acids and specific purification measures need to be used to remove these contaminants from the nucleic acids. Further, the abundant nuclease enzymes that are released upon cell disruption degrade DNA and RNA rapidly unless provision is made to reduce enzyme activity by the presence of specific inhibitory compounds or by the reduction of temperature.

[0004] In many procedures that are current art, genomic DNA is directly isolated [Csaikl et al., 1998, Plant Molecular Biology 16:69-86], free plant DNA is liberated into detergent or chaotropic buffer solution directly during or immediately following the tissue homogenization step, and the DNA is then purified by partition from denatured cell debris and proteins by sequential extraction steps using aqueous-phenol or aqueous-chloroform. The organic solvents cause strong denaturation of most proteins and polysaccharides and dramatically reduce their solubility in aqueous buffers. This denaturation effect, accompanied by the partitioning of DNA to the aqueous phase and the denatured cell materials to the interface between phases, achieves the "purification" in these two-phase systems. In the present art, a high concentration of salt [NaCl, KCl] is used to salt out the contaminating proteins, or to promote differential solubility of polysaccharides and DNA during subsequent stages of ethanolic precipitation of DNA for recovery of DNA from aqueous solution. Multiple, sequential two-phase extraction steps are usually necessary to remove residual contaminants. The repeated extraction steps may result in excessive DNA shear during phase mixing and during the collection of the separated aqueous phases by pipette or similar devices.

[0005] The mechanical shear forces necessary for tissue disruption and DNA release may itself be a cause of the rupture of plant organelles and for direct DNA breakage. Further, during strong mechanical shear, plant cell nucleases may also be liberated and cause extensive degradation of plant nucleic acids. Hence, plant cells are frequently disrupted

under conditions of low shear, such as by grinding in a mortar and pestle under liquid nitrogen [-196 °C]. These conditions prevent all enzyme activity, and aid cell fracture as membrane liability is low, while the cellulose walls are brittle and fragile and are easily broken. Although, the extreme cold affords high protection to nucleic acids and proteins, and the shear generated during grinding is low, the procedure is not particularly amenable to automation and is usually a low-throughput, manual procedure.

[0006] Per-Åkke Albertsson and others have developed methods for the partial purification of free cells and of cell organelles [Albertsson 1970, Advances in Protein Chemistry 24:309-341; Albertsson 1986, Partition of cell particles and macromolecules, pp. 147-172; Albertsson 1988, Quarterly Review of Biophysics 21:61-98; Albertsson 1989, Methods in Enzymology 171:532-549; Persson and Jergil 1995, Federation of Associated Societies for European Biochemistry: Journal. 9:1304-1310] by exploitation of the differential compatibility of cells and cell organelle membrane systems with a particular phase medium within an aqueous phase system comprising two or more resolvable polymer phases. Phase partition resulting in the concentration of the cell or cell organelle into a particular phase medium, or to the interface region between the two phases. The approach taken was to exploit the partition behaviour to provide a marked enrichment of a particular desired cell type or cell organelle into a particular phase medium, accompanied with a marked diminution in the amount of one or more other cell types or cell constituents from that same particular phase medium.

[0007] Further, Albertsson and others have developed methods for the partial purification of free DNA [Albertsson 1986, Partition of cell particles and macromolecules, pp. 147-172; Albertsson 1988, Quarterly Review of Biophysics 21:61-98; Albertsson 1989, Methods in Enzymology 171:532-549; Ohlsson et al., 1978, Nucleic Acids Research 5:583-590] by exploitation of the differential compatibility of nucleic acids with a particular phase medium within an aqueous phase system comprising two or more resolvable polymer phases. Phase partition resulting in the concentration of the DNA into a particular phase medium.

[0008]Although aqueous phase partition of solutes and particles is well known to those practiced in the art of particle separations, it is not currently employed as the medium for purification of cell nuclei or for DNA purification in any commercial apparatus employed for the purification of DNA from any organism, bacteria, single cell, or solid tissue.

[0009]With the foregoing in view, this invention in one aspect resides broadly in a method of isolating cell nuclei from a tissue sample including the steps of:

- placing said tissue sample in a nuclease inhibiting buffer;
- tissue-milling said tissue sample and buffer under low shear conditions to release cell nuclei from the tissue;
- gravimetrically concentrating said nuclei at the boundary layer of differential density media, and
- collecting said nuclei-rich boundary layer.

[0010]Preferably, the foregoing method is conducted in a continuous manner. The tissue sample may include tissue from plants, animals, fungi or bacteria. Preferably, this tissue is from a plant.

[0011]The tissue may be milled by an automated tissue mill under low shear conditions for the liberation of high yields of "high quality" cell nuclei. By "low shear conditions" it is meant that the conditions are whereby disruption of the tissue and its cells occurs without substantial degradation of the cellular organelles. The differential density media may be selected to partition the nuclei from the milled tissue at a boundary layer formed thereby. For example, the media may include the polymers dextran and polyethylene glycol (PEG). Alternatively, additional or alternative compounds may be included or substituted for the forgoing compounds. For example, alternative aqueous phase forming systems may be Ficol:Dextran, Uncon:Dextran, PVP/PEG:Dextran, PVP:PEG or Salt:PEG, or some combination of an polymer with a substituted-Dextran, or a substituted-PEG polymer with Dextran.

[0012] Any suitable buffers may be selected. For example, the buffer may be potassium phosphate, preferably in the range of pH 7.0 - 8.0. Agents may be included in the buffer that may act to protect the integrity of the nuclei and to protect DNA integrity from the activity of nuclease enzymes. Agents that may inhibit oxidation processes that promote the polymerization of reactive phenolic compounds may also be included in the buffer. These additives may include soluble polyvinylpyrrilidone (PVP), EDTA and dithiothreitol or β-mercaptoethanol, a non-ionic detergent such as Triton or Tween or a combination thereof. It is to be understood that the foregoing additive compounds are not exclusive, and that additional or alternative compounds could be included or substituted for the forgoing compounds.

[0013] The rapid resolution of the differential density media to partition of the free nuclei to the interphase region between the phases may be achieved by the exertion of centrifugal or acoustical forces on the system. The external force may be achieved by centrifugation of the phase system, or by transduction of ultra-sound through the phase system mixture.

[0014] Motor-driven mills [ball or bead mills] and tissue homogenizers and blendors [Blade mills, Waring blender, Ultra-Turrax, Polytron homogenizer] and high speed centrifugal blade mills [juice extractors] have also been frequently used for convenience and to avoid manual processing of tissues [Loomis 1973, Methods in Enzymology 31:528-545; Price 1968, Methods in Enzymology 13:501-525; Willmitzer and Wagner 1981, Experimental Cell Research 135:69-77]. Although some mechanical milling procedures have employed at low or extreme frozen temperatures that inhibit all oxidative and enzymatic activities until the addition of a protective buffer to the ground tissue, many reports have allowed tissue disruption in the presence of protective buffer [wet milling], during which the relatively rapid speed of the homogenization process and infiltration of the buffer affords some protection to the nucleic acids. Almost all mechanical milling procedures are subject to higher shear than manual homogenization, and some shear damage inevitably occurs to nuclei and cell

organelles [Spelsberg et al., 1984, Analytical Biochemistry 143:237-248], or to the liberated chromosomal DNA. Ball mills are particularly hard to control reproducibly, particularly with the quality of DNA isolated from woody tissues as the extensive agitation necessary to release DNA may readily shear it. The isolation of full length RNA is generally more reproducible than DNA as it is less subject to shear effects because of its lower molecular weight [Eggermont et al., 1996, Plant Molecular Biology Reporter 14:273-279].

[0015] Although rollers and crush mills were favorably recommended in early reports [Loomis 1968, Methods in Enzymology 13:555-563; Loomis 1973, Methods in Enzymology 31:528-545], the loss of extraction buffer or the inconvenience in their operation has led to their replacement by motorized homogenizers and cutting devices such as the Polytron, UltraTurrax and Waring blendors. Further, the extensive homogenization required for the release of large organelles such as cell nuclei, or for the efficient disruption of tissues from tough and fibrous plant species led to the adoption of mechanical tissue homogenizers for large scale preparations. Notably, although motorized high-speed homogenizers have conspicuous advantages such as high throughput of tissue and rapid operation, there is often appreciable damage to cell organelles or extensive liberation of biopolymers that necessitate lengthy downstream purification steps, in addition to the removal of cell debris.

[0016] Accordingly, in another aspect this invention resides in a tissue mill including:

- a rotor and stator having complementary conical surfaces, each surface having one or the other of complementary shearing elements including a plurality of knife edges arranged on one said surface and substantially lying in a radial plane of said surface, and a helical knife edged conduit disposed on the other of said surfaces.

[0017] The stator may be located within the rotor. However, it is preferred, the rotor is located within the stator. It is also preferred that the stator includes the plurality of knife edges, while the rotor has the helical knife edged conduit. In this arrangement, the tissue may flow along the knife edged conduit of the rotor and as the plurality of knife edges of

stator pass by the tissue is able to move and rotate, being chopped into small pieces as the two knife edges pass.

[0018] A suitable buffer may be fed into the mill to ensure to ensure flushing of the mill during operation. It is preferred that the buffer is fed continuous during operation. The continuous provision of buffer solution during milling may provide protection for the DNA that remains within the cell nucleus and may prevent tissue blockage.

[0019] The mill may also include a feeder to deliver the tissue to the rotor. For example, the feeder may be a helix. The helix may also include knife-edges, so that as tissue is fed into the rotating helix it is chopped to a size to assist in the milling stage at the rotor and stator.

[0020] The surfaces of the mill, in particularly the helix, rotor and stator may be coated in a suitable metal surface treatment and coating. This may increase wear and reduce friction. The coating may also prevent tissue clogging and to assist in cleaning away tissue left behind after milling. The surface treatment may include titanium nitride, in particularly by the commercial Balinit process.

[0021] Following milling of the desired sample of tissue the mill may flushed with a washing solution leading to waste to prevent mixing of nuclear material between different tissue samples. Parts of the mill, such as rotor, stator and helix may be adjusted to allow cleaning of tissue, which may adhere to the mill, in particular in any crevices.

[0022] The clearance between the rotor and stator may be adjustable to adjust the size of the milled tissue at its output. Also, this adjustable clearance may ensure that soft tissue and tough tissue of woody plants may be effective broken to release nuclei in sufficient yields.

[0023] The clearance between the rotor and stator may also be varied across the length of the mill, such that following delivery of tissue into the posterior [narrow end] of the mill there may be sufficient clearance for the partially intact plant tissues. The forward movement of the tissue towards the anterior [wide end] of the mill and corresponding commination of the tissue may occur across the mill working surfaces in which the clearance between the rotor and stator may diminish until a set minimum clearance between rotor and stator is achieved.

This minimum clearance may be constant across the remaining portion of the mill working surfaces, providing for fine milling of the plant tissues prior to exit from the mill. The volume available for occupation by the plant tissue may be relatively constant across the length of the mill. This may be provided by both the increasing surface area of the conical rotor and stator and the increasing width in the spacing between the helically aligned knife-edges on the rotor and radially aligned knife edges on the stator.

[0024] Centrifugation is often used to accelerate the resolution of phase systems and the partition of solutes [Albertsson 1986, Partition of cell particles and macromolecules. pp. 102-111; Cole 1991, BioTechniques 11:18-24; Miller 1994, Methods in Enzymology 228:193-206; Lantz et al. 1996, Journal of Chromatography: Biomedical Applications 680:165-170] and particles [Albertsson 1970, Advances in Protein Chemistry 24:309-341; Albertsson 1989, Methods in Enzymology 171:532-549; Hernandez et al., 1996, Journal of Chromatography: Biomedical Applications 680:171-181] contained within the systems.

[0025] Although the use of flow centrifugation for the resolution of aqueous phase systems is known to those practised in the art [Sutherland and Ito 1980, Analytical Biochemistry 108:367-373; Ito and Zhang 1988, Journal of Chromatography 437:121-129; Shinomiya et al., 1993, Journal of Chromatography 644:215-229] the design of such apparatus is substantially based on a planetary centrifuge which holds a separation column consisting of multiple (about 200) partition units connected in series with transfer tubes. The phase systems exchange solutes by virtue of a counter current mixing process in which aqueous phase elements are mixed and separated within the different partition units. Although phase separations are highly effective, the apparatus is not compatible with high throughput application and is more suited to partition of compounds with small differences in partition coefficient that demand numerous repeated partition reactions.

[0026] Further, flow centrifuges [US4217418, US5641622, US5722926, US5618409, US5169377, US4252020; US6238330; US6273848, US6440054], mixers [US6431745] and

compartmented flow centrifuges [US4543085, US5610074, US5746789] are known to those practised in the art for the continuous separation of solutes, particles or cells from continuously inflowing density partitioned solutions or suspensions of particles in which the centrifugation process accelerates the separation or compartmentalisation of the object particle or solute containing density solution and directs a continuous outflow of solution that is partitioned, has enriched density, or is a particle suspension. These applications of the aforementioned continuous flow centrifugation apparatus and methods are not amenable to the purification of small batches of particles from discrete individual tissue samples.

[0027] Accordingly, in a further aspect this invention relates to a method of separating nuclei from cellular material including the steps of;

- forming a mixture of said cellular material in a differential density medium selected to partition said nuclei at a boundary layer formable thereby;
- centrifuging said mixture in an annulus of a rotor to partition said mixture into discrete density media and
- removal of the nuclei-rich boundary layer of said partitioned media whilst the rotor is rotating.

[0028] The design of the centrifuge rotor and manner of collection of the soluble and partitioned media are of particular importance for this purpose. An annular rotor, which provides for a high surface to volume ratio allows the partition of said nuclei into a dense band that occupies a minimal radial thickness.

[0029] The said phase media and the said nuclei may be collected independently by a tubular arm that siphons the separated phase mixture solutions from the top of the rotating phase mixture [inner radius surface of minimum *g*] during continuous low-speed centrifugation. The tubular arm may be positioned close to and above the inner surface of the slowly centrifuging solution, perpendicular to the surface of the solution, or at a tangent or at another appropriate angle between these angles to the liquid surface and the phase solutions and the band of nuclei are conveniently collected by means of a pump or vacuum

action exerted on the tubular arm or by the centrifugal action of the liquid alone. Most conveniently, the liquid is collected by an active pump action exerted on the tubular arm. The fraction of collected solution containing the nuclei or other fractions may be monitored by various means such as optical density or light scattering or video image, or by reference to a predicted position of each fraction in the annular rotor or by any other means of identification. The invention is not limited to the application here demonstrated for the purification and fractionation and collection of an enriched cell nuclei fraction but may be applied to the purification and fractionation and collection of any liquid, particulate or polymeric substance that may be purified and fractionated and subsequently collected following centrifical fractionation whether in phase media or other liquid or polymeric media. Examples of other particulate and cellular species that may be partitioned by such centrifugal fractionation using phase solutions might include blood cells including nucleated white cells [Latham et al, 1969, Vox Sang 17:19; Crandall et al, 1973, Minnesota Medicine 56:759-761; Runck et al, 1972, Transfusion 12:237-244; Pietersz et al, 1990, Vox Sang. 59:205-208; Malferrari et al, 2002, Biotechniques. 2002 33:1228-1230], cellular organelles [Leighton et al, 1968, Journal of Cell Biology 37:482-513] or cells from animal tissues [Rodnight et al, 1969, Journal of Neurochemistry 16:1581-1589; Villani et al, 1994, Transplant Proceedings 26:621-622; Chern et al, 1993, Proceedings of the National Science Council of the Republic of China B 17:143-151].

[0030] The differential density medium may include mixture of polymers including dextran and polyethylene glycol (PEG). Alternatively, additional or alternative compounds may be included or substituted for the forgoing compounds. For example, alternative aqueous phase forming systems may be Ficol:Dextran, Uncon:Dextran, PVP/PEG:Dextran, PVP:PEG or Salt:PEG, or some combination of an polymer with a substituted-Dextran, or a substituted-PEG polymer with Dextran.

[0031] The cellular material may be tissue that has been milled to a suitable size to release the nuclei from the tissue. Preferably, the tissue has been milled under low shear conditions.

Centrifugation may provide for more rapid isolation of nuclei from discrete samples of homogenates containing cellular material. The above method may function for batch processing of discrete tissue samples in a continuous process cycle.

[0032] The tubular phase collector may be inserted into the phase mixture within the centrifuge rotor during low speed rotation, to provide for turbulent mixing of the differential density medium and the cellular material prior to centrifugation.

[0033] Alternatively, the centrifuge rotor may have one or more small mixer/agitator vanes at the base of the bowl of the rotor which may provide for low speed rotation for mixing of the differential density medium and the cellular material, prior to centrifugation.

[0034] The partitioned media may be collected any suitable means. For example, a collecting arm including a conduit or siphon may be is lowered into the surface of the phase solution during operation of the centrifuge. The desired partition of the media may be drawn by suction into the arm or may be actively collected by a motorised pump. The partition collected may be monitored spectrophotometrically to monitor its composition. According, to its content the collected partition may be collected for further processing, such as the boundary layer, or removed to waste.

[0035] The position of the collection arm may be controlled automatically by any suitable means. For example, a position motor may actuate movement of the collection arm in both vertical and rotational movement. The arm may be inserted and retracted from the centrifuge bowl at appropriate times.

[0036] The speed of rotation of the centrifuge may be reduced to an angular velocity commensurate with the retention of the partitioning of the density media in the annular collection chamber.

[0037] The rotor may also provide for "through-flushing" and cleaning of the centrifuge and attendant apparatus prior to the processing of the subsequent sample in a continuous cycle of action. To assist in cleaning the collection arm may have one or more washing devices,

such as spray nozzles, to clean the surface of the centrifuge rotor before introduction of the subsequent tissue sample.

[0038] Optical sensing devices may also be placed it the path of the siphoned solution are used to detect the different fractions of the phase solution and processor controlled valves allow for routing of particular sample fractions for preservation or to waste.

[0039] Centrifugation is also used to accelerate the resolution of phase systems and the partition of solutes [Albertsson 1986, Partition of cell particles and macromolecules. pp. 102-111; Cole 1991, BioTechniques 11:18-24; Albertsson 1989, Methods in Enzymology 171:532-549] contained within the systems. Accordingly, in a further aspect this invention relates to a method of separating deoxyribose nucleic acid from cellular material including the steps of;

- forming a mixture of said cellular material in a differential density medium selected to partition said nucleic acid at a lower layer formable thereby;
- centrifuging said mixture in an annulus of a rotor to partition said mixture into discrete density media, and
- removal of the deoxyribose nucleic acid-rich bottom layer of said partitioned media whilst the rotor is rotating.

[0040] The design of the centrifuge rotor and manner of collection of the soluble and partitioned media are of particular importance for this purpose. An annular rotor, which provides for a high surface to volume ratio allows the partition of said deoxyribose nucleic acid into a discrete phase that occupies a defined radial position in the separated phases.

[0041] The said phase media and the said deoxyribose nucleic acid may be collected independently by a tubular arm that siphons the separated phase mixture solutions from the top of the rotating phase mixture [inner radius surface of minimum g] during continuous low-speed centrifugation.

[0042] The said phase media and the said deoxyribose nucleic acid may be collected independently by a tubular arm that siphons the separated phase mixture solutions from the top of the rotating phase mixture [inner radius surface of minimum *g*] during continuous low-speed centrifugation. The tubular arm may be positioned close to and above the inner surface of the slowly centrifuging solution, perpendicular to the surface of the solution, or at a tangent or at another appropriate angle between these angles to the liquid surface and the phase solutions and the nucleic acid containing phase are conveniently collected by means of a pump or vacuum action exerted on the tubular arm or by the centrifugal action of the liquid alone. Most conveniently, the liquid is collected by an active pump action exerted on the tubular arm. The fraction of collected solution containing the nucleic acid or other fractions may be monitored by various means such as optical density or light scattering or video image, or by reference to a predicted position of each fraction in the annular rotor or by any other means of identification. The invention is not limited to the application here demonstrated for the purification and fractionation and collection of an enriched cell nuclei fraction but may be applied to the purification and fractionation and collection of any liquid, particulate or polymeric substance that may be purified and fractionated and subsequently collected following centrifical fractionation whether in phase media or other liquid or polymeric media.

[0043] The differential density medium may include mixture of polymers including dextran and polyethylene glycol (PEG). Alternatively, additional or alternative compounds may be included or substituted for the forgoing compounds. For example, alternative aqueous phase forming systems may be Ficol:Dextran, Uncon:Dextran, PVP/PEG:Dextran, PVP:PEG or Salt:PEG, or some combination of an polymer with a substituted-Dextran, or a substituted-PEG polymer with Dextran.

[0044] Coakley et al. [1994, Bioseparation 4:73-83] have described the manipulation of particles and cells within aqueous phase systems by the application of high frequency

ultrasound and several patents refer to the manipulation of cells and particles in solution by ultrasound [US5527460, USP5484537]. Although the use of ultrasound to facilitate the concentration and migration of cells and small particles from bulk fluid, the efficacy of ultrasound for the manipulation and concentration of free cell nuclei in an acoustic medium is not well understood.

[0045] Accordingly in one aspect this invention resides in a method of separating nuclei from cellular material including:

- forming a mixture of said cellular material in a differential density medium selected to partition said nuclei at a boundary layer formable thereby;
- inducing standing waves in said mixture to partition mixture into discrete nodal density media bands including said boundary layer between discrete density media bands, and
- removal of said nuclei-rich boundary layer.

[0046] The cellular material may be tissue which has been milled to a suitable size to release the nuclei from the tissue. Preferably, the tissue has been milled under low shear conditions.

[0047] The differential density medium may include mixture of polymers dextran and polyethylene glycol (PEG). Alternatively, additional or alternative compounds may be included or substituted for the forgoing compounds. For example, alternative aqueous phase forming systems may be Ficol:Dextran, Uncon:Dextran, PVP/PEG:Dextran, PVP:PEG or Salt:PEG, or some combination of an polymer with a substituted-Dextran, or a substituted-PEG polymer with Dextran.

[0048] The mixture may be contained in a suitable receptacle which to allow conduction of resonance. The receptacle may include an inlet and outlet for the mixture and for the collection of the resolved particles. The receptacle may be ultrasound reflective vessel to accelerate the resolution of the mixture and provide for the migration of the nuclei to the boundary region between the differential density media. For example, the receptacle may be

made of a thin wall plastic, such as polyallamer. A reflective acoustic material, such as plastic or glass may be included to reflect the standing waves to assist resolution.

[0049] The standing waves may be generated by any suitable means. For example, a piezoelectric force transducer may be provided with electrodes and is activated by appropriate RF amplifier. Alternatively, two piezoelectric force transducers may be provided and driven at different frequencies to provide for the directed movement of the nuclei particles towards one of the transducers. The transducer may be of any suitable material that allows transmission of the piezoelectric force into a mechanical oscillation. For example the transducer may be ceramic. The active piezoelectric force transducer may apply mechanical oscillation to the mixture at a resonance frequency of approximately 1MHz.

[0050] Not being bound by theory, it is supposed that under the influence of ultrasound Lamb-wave forces many nuclei (acting as particles) aggregate together into large particles over small distances. These particles then migrate to the interface region that occur at the standing minima caused by the superimposition of active and reflected ultrasonic waves.

[0051] Subsequent to the resolution of the mixture, the boundary layer may be collected from the settled phases by pumping the resolved solutions out of the separation chamber. Optical detection may assist in the location of the nuclear material at the interface the nuclei-rich boundary which then can be collected separated from the bulk of the partitioned media.

[0052] The partition of nuclei in a mixture exposed to ultrasonic standing waves may limit any damage or alteration to the length of the DNA contained within the nuclei aggregates. This may provide for a rapid and convenient method for the concentration and partial purification of nuclei from a crude filtered homogenate of tissues.

[0053] Cole and colleagues [US5611904, US5707850] have described the use of electrophoresis to concentrate long DNA molecules by a method of electrochromatography in which the migration of the DNA under electromotive force is opposite to the motion of bulk laminar fluid flow. Using appropriate rates of fluid flow and applied electric field, the DNA

can be retained at a virtual stationary position within the flow cell matrix while bulk fluid is moved through the cell and exchange of DNA-depleted by DNA-rich solution may occur, allowing the concentration of DNA within the flow cell.

[0054] Further, Tomblin and colleagues [US4750982] have described a process for purifying and concentrating genomic DNA from blood in an apparatus which consists of a disc of high purity agarose gel, immersed in an electrophoresis buffer solution and supported between two polycarbonate filters in an electric field. The genomic DNA is concentrated within the agarose disc and other components of a blood lysis pass through the gel disc. While the method describes that DNA can then be electroeluted by further application of the electric field and collected in concentrated form, the apparatus is essentially for slow manual operation and requires that the DNA be partially sheared to prevent damage to the agarose gel disc.

[0055] Pure silica, finely divided glass and diatomaceous earth have also been widely employed for the purification of small DNA molecules such as bacterial plasmids and restriction fragments of DNA. Affinity and ion-exchange methods are also widely used for the purification of nucleic acids from biological sources. However, Müller [1986, European Journal of Biochemistry 155:203-212] showed that several commercial ion-exchange resins which are widely utilized for purification of plasmids and DNA restriction fragments have severe limitations on for the recovery of DNA fragments longer than several kilobasepairs (kb). In addition, the binding of long DNA molecules to silica surfaces is virtually irreversible and only low recovery can be anticipated even using alkaline buffer of low ionic strength to elute long genomic DNA.

[0056] Further, several commercial kits and methods for DNA purification employ surface-modified silicas which do not bind DNA but rather bind cellular contaminants such as protein and polysaccharides from the solution containing the DNA. Although such surface-modified silicas are effective in purifying genomic DNA from most plant species and overcome the need to elute DNA from a binding-matrix, some modified polysaccharides present in plants

are not readily removed by these matrices and repeated extractions are required to ensure complete removal of the polysaccharide from the DNA. In addition, potentially toxic organic solvents are employed with such surface-active matrices to drive the interactions between the silica-bound ligand and the contaminant polymer.

[0057] Several authors [Bünemann and Müller 1978, Nucleic Acids Research 5:1059-1074; Müller et al. 1981, Nucleic Acids Research 9:95-119; Koller et al. 1978, Gene 4:227-239, Vacek et al. 1982, Analytical Biochemistry 124:414-420; Fedorov et al., 1984, Biokhimiia 49:1708-1711] have described the attachment of planar aromatic compounds such as malachite green, fuchsin, phenyl neutral red, phenyl phenazinium dye, ethidium or Hoechst 33258 which display preferential binding for particular DNA sequences, or particular base pair regions in DNA. Such affinity dyes were attached to a variety of solid matrices such as polyacrylamide and PEG that act to retain and bind DNA to a solid surface. In the foregoing, cacodylate, sodium chloride or elevated temperature was utilized to modulate the release of bound DNA from the affinity resins, with differing efficiencies influenced by length of the DNA molecule, the base composition of the DNA and the particular affinity dye resin.

[0058] Further, Ford and colleagues [US4623463, US4661526, US4970273, US5141611] have described polymeric porous membranes and solid materials on which a surface chemistry may be modified by glutaraldehyde and carbodiimide treatments for the purpose of attachment of active affinity groups to that surface.

[0059] Accordingly, in still further aspect this invention resides in apparatus for separating charge molecules including:

- a chamber having an inlet and an outlet for a solution of said charge molecules;
- a semipermeable barrier disposed in said chamber and serving to separate a selection constituent for said charge molecules from said chamber, and

- an electric field adapted to cause migration of said charge molecule across said barrier and into engagement with said selection constituent.

[0060] The charged molecule may include any nuclear material, such as DNA or RNA, or other biomolecules such proteins carbohydrates. It has in particularly been determined that the above apparatus is suitable for the separation of DNA. Accordingly, the following examples are given in respect of DNA. However, it is to be understood that a person skilled in the art would be able to make the appropriate substitutions to enable separation of the molecules of interest.

[0061] The semipermeable barrier may be semi-porous membrane which is selectively permeable to the molecule of interest. For example, this may include cellophane or dialysis membrane. Alternatively, the selection constituent may form a semipermeable barrier by the nature of the substance. For example, the selective constituent may be solid.

[0062] The selective constituent may be any suitable medium which may select the molecule of interest by any suitable means. For example, the selection may be based on size selectivity, such as in gels or sieving media, or by attracting and selectively binding the molecule of interest, such as in affinity matrices.

[0063] Accordingly, the selective constituent may include gels such as agarose and sieving media such as an entangled form of solutions. For example, these solutions may include liquid agarose polymer, or soluble cellulose polymers such as hydroxypropyl methyl cellulose and hydroxyethyl cellulose, linear polyacrylamide, linear N-acryloylaminoethoxyethanol, or very high molecular weight dextran. The selective constituent may also include also employ a particulate bed or affinity matrix resins in which an affinity-dye may direct the binding. For example, ethidium, Hoechst 33258 or fuchsin may be utilised via primary amine or quaternary amine functions respectively to the hydroxyl groups on the surface of a polyacrylamide particle, or sepharose particles, or sulfonyl chloride resin, surface activated nylon, or the surface of fine silica dioxide particles, or onto paramagnetic particles possessing a surface coating of silica, polyacrylamide or using a glutaraldehyde linker

moiety. A wide variety of microparticle supports may be used with this invention, including microparticles made of controlled pore glass (CPG), acrylic copolymers, cellulose, nylon, dextran, highly cross-linked polystyrene, polyacrolein and the like, disclosed in the following references: Methods in Enzymology, Section A, pages 11-147, vol. 44 (Academic Press, New York, 1976); US Patent Nos. 4,678,814; 4,413,070 and 4,046,720; and 5,141,611; and Pon, Chapter 19, in Agrawal, editor, Methods in Molecular Biology, vol. 20, (Humana Press, Totawa, N.J., 1993). Glutaraldehyde is a well known cross-linking or functionalizing agent capable of covalently attaching amines [such as proteins] to solid supports [Komissarenko and Avrameas, (1978) Ukr Biokhimika Zh 50:500-511; Timofeev et al. (1996) Nucleic Acids Research 24:3142-3148; Sano et al. (1993) Biomaterials 14:817-822; Panikkar et al. (1997) Artificial Cells and Blood Substituent Immobilization Biotechnology 25:541-550; Prabhune and SivaRaman (1991) Applied Biochemistry and Biotechnology 30:265-272; Kelleher and Juliano (1984) Analytical Biochemistry 136:470-475; Guesdon and Avrameas (1976) Journal of Immunological Methods 11:129-133; Karey and Sirbasku (1989) Analytical Biochemistry 178:255-259, US5141611]. Also, an ethidium-acrylamide affinity resin or Hoechst 33258-acrylamide affinity resin may be used for DNA binding in which the ethidium or Hoechst 33258 is attached to co-polymerizing acrylamide and bisacrylamide polymers to form an affinity surface, or a polyacrylamide particle [Bünemann and Müller 1978, Nucleic Acids Research 5:1059-1074; Müller et al. 1981, Nucleic Acids Research 9:95-119; Koller et al. 1978, Gene 4:227-239, Vacek et al. 1982, Analytical Biochemistry 124:414-420; Fedorov et al., 1984, Biokhimiia 49:1708-1711]. However, it is to be understood that the foregoing acrylamide-based affinity resins and DNA-binding silica matrices are not exclusive, and that additional or alternative compounds could be included or substituted for the foregoing compounds.

[0064] Free DNA may be concentrated from the solution into the selective constituent by application of an electric field perpendicular to the direction of bulk laminar fluid flow. Other molecules that possess an opposite net charge to DNA or net neutral charge will either

electromigrate towards the cathode in the opposite direction to the migration of the DNA, or will not electro-migrate respectively, and thus will reduce contact of such contaminant biomolecules with the affinity matrix. The selective constituent may reduce the electrophoretic migration of the DNA relative to its migration in free solution, allowing the nucleic acid to accumulate in the selective media. This may allow fresh DNA containing solution to may replace DNA-depleted solution until a saturation of dye-binding sites is achieved

[0065] The molecule of interest may be recovered directly from the selective constituent by any suitable procedure appropriate to the media. For example, if the selective constituent is an affinity matrix the molecule may be released by alteration of the ionic environment by an alkali salt of the halide series such as KI, NaI or KCl following recovery of the matrix from the flow cell or the electroretention cell. Alternatively, if the media is a gel, the solution in the chamber may be replace by a fresh buffer and the polarity of the electrodes reversed to release the molecule back in the fresh buffer.

[0066] In a further embodiment, the selective constituent may be mobile, so that the procedure can be continuous. Removal of the desired molecule may be retrieved subsequently.

[0067] Many devices and methods able to achieve for each aspect of plant cell breakage and subsequent isolation of plant DNA have been described, though none of these devices or methods provides for automated isolation of genomic DNA from plant tissues. Indeed, although several "plant DNA isolation kits" are commercially available [Colvin et al., 1995, Biochemica 3:12-13; Csaikl et al., 1998, Plant Molecular Biology 16:69-86; Lee and Nicholson 1997, Nature Biotechnology 15:805-806; Qiagen – DNeasy Plant System; Boehringer-Mannheim – Plant DNA Isolation kit] these modern procedures also depend upon prior disruption of plant tissue, or require extensive and numerous manual steps to obtain pure DNA which is typically 50 kb or shorter because of mechanical shear imposed during

the purification stages, also because the ion exchange resins used for DNA final steps of purification preferentially release shorter DNA fragments [Müller 1986, European Journal of Biochemistry 155:203-212]. Further typically the present art employs particular steps to immobilize plant cells or plant cell nuclei in solid gel media may be employed to prevent exposure of liberated DNA to shear forces [Lui and Whittier 1994, Nucleic Acids Research 22:2168-2169; Luro and Laigret 1995, BioTechniques 19:388-392; Zhang et al., 1995, The Plant Journal 7:175-184]. In addition, both the traditional methods and "plant DNA isolation kits" often use steps involving harsh chemicals or organic solvents to protect the DNA from degradative enzymes or to denature and remove high and low molecular weight plant compounds that could be contaminate the free DNA.

[0068] Also, although a number of semi-automatic or fully automatic DNA purification machines are commercially available, their principal focus is the purification of plasmid DNA or phage DNA from bacteria [Qiagen - BioRobot 9600; Precision System Science - SX-κg; Integrated Separation Systems - AutoGen P850; Applied Biosystems - GenePure 341; Talent - Extragen 8C]. Several automated DNA purification machines are also able to purify DNA from fluid or single cell tissues such as blood and cell suspensions [Qiagen - BioRobot 9600; Precision System Science - SX-κg; Toyobo - MFX2000; Integrated Separation Systems - AutoGen P850; Applied Biosystems - GenePure 341; Talent - Extragen 8C].

[0069] The aforementioned systems either utilize the binding of DNA to particles of silica [Precision System Science - SX-κg; Toyobo - MFX2000; Talent - Extragen 8C] or the binding of DNA to ion-exchange binding to anion-binding particles and resins [Qiagen - BioRobot 9600] or to anion-binding membrane filters [Pharmacia - EasyPrep System] [Mack 1996, The Scientist 10:17-18]. Several systems are based on classical adaptations of laboratory methods including centrifugation and organic solvent extraction [Applied Biosystems - GenePure 341; Tetrad - Centrifugal DNA Extractor; Integrated Separation Systems - AutoGen]. Although for several machine protocols have been developed for isolation of genomic DNA from solid mammalian tissues and from plant tissues [Anon 1996, Qiagen

News 5:8-9; Rogers et al. 1996, Strategies Newsletter 9:73-74; Koepf et al., 1992, Plant Genome Conference I; Integrated Separation Systems - AutoGen P850; Precision System Science - SX-κg] the tissue first needs to be independently homogenized and then the clarified homognate is introduced into the DNA isolation machine for final steps to purify the DNA from residual contaminants.

[0070] Accordingly, the invention in one aspect resides broadly in a method for the isolating and purifying nuclear material including:

- milling tissue under low shear conditions to release cell nuclei;
- resolving said cell nuclei;
- digesting nuclear membranes of cell nuclei to release free nucleic acids;
- resolving said digest to separate partition released nucleic acid and
- collecting said nucleic acid partition, and
- purifying said nucleic acid-rich partition.

[0071] In a further aspect, this invention in resides broadly in a method of purifying long genomic DNA from "high quality" plant cell nuclei following the mechanical disruption of plant cells by the use of a series of aqueous two-phase partition procedures, including the steps of:

- collection of plant tissue in a array sampler for automated delivery into the automated tissue mill.
- release of plant cell nuclei using low-shear milling equipment and methods of wet milling.
- thorough mixing of homogenised plant tissue and homogenisation buffer.
- centrifugal resolution of a mixture of aqueous two-phase polymers and a plant cell homogenate.
- automatic collection of a nuclei-rich fraction of the resolved phase mixture.

- protease digestion of nuclear membranes to liberate free DNA.
- centrifugal resolution of a mixture of residual aqueous two-phase polymers and a plant DNA containing solution.
- affinity binding of the free DNA solution to a solid affinity matrix with high specificity for DNA.
- release of pure DNA from the affinity matrix by exposure to salts or agents that affect quantitative release of DNA.
- alcohol precipitation of DNA from aqueous solution and collection of the denatured DNA by vacuum filtration.

[0072] In order that this invention may be more readily understood and put into practical effect, reference will now be made to the following figures and examples which illustrate preferred embodiments of the invention and wherein:

- FIG. 1 is a field linear tissue sampler;
- FIG. 1A is a sample tube in the tissue sampler;
- FIG. 2 is a field array tissue sampler;
- FIG. 3 is a plan view of the automatic delivery of the tissue sampler into the tissue mill;
- FIG. 3A is a three-dimensional view of the automatic delivery of the tissue sampler as illustrated in FIG. 3;
- FIG. 4 is a plan view of the tissue mill;
- FIG. 4A is a three-dimensional view of FIG. 4
- FIG. 5 is views of the rotor of the tissue mill;
- FIG. 6 is views of the stator of the tissue mill;
- FIG. 7 are plan views of the post-homogenisation mixing chamber;
- FIG. 8 is the plan view of the flow centrifuge;

- FIG. 8A is the three dimensional view of the flow centrifuge as illustrated in FIG. 8;
- FIG. 9 is cross sectional view of the flow centrifuge including the collection arm;
- FIG. 10 illustrates alternative designs of the agitator/mixer of the flow centrifuge rotor;
- FIG. 11 is an open-top view of the flow centrifuge from above, depicting devices for the collection of the partitioned solution
- FIG. 11A is a washing head spray;
- FIG 11B is a probe siphon;
- FIG 12 depicts one configuration of a piezoelectric force transducer to apply mechanical oscillation to the aqueous two-phase system;
- FIG. 13 is an electric flow cell;
- FIGS 14 to 18 are alternate flow charts for automated DNA purification;
- FIG. 19 is the helix of the tissue mill;
- FIG. 20 is the side perspective of the rotor of the tissue mill illustrating the tooth;
- FIG. 21 is the opposite side of rotor of the tissue mill from Fig. 20;
- FIG. 22 is the stator of the tissue mill;
- FIG. 23 is a perspective view of the helix and rotor of the tissue mill;
- FIG. 24 is side view of the helix of the tissue mill, and
- FIG. 25 is a side perspective view of the helix and rotor of the tissue mill

EXAMPLE 1

Tissue sampler

[0073] The tissue sampler is a convenient means for the collection of sufficient tissue in the field into a device which, in one configuration of the device, provides for the recording of individual tissue samples into a particular sample cell of a linear sampler, as illustrated in one embodiment in FIG. 1 and 1A.

[0074] Referring to FIG. 1, the tissue sampler [1-1] includes a slide lid [1-2], slide base [1-3] and sample tubes [1-4] which are open top and base. The tubes are attached to parallel grooved walls [1-5] which act as cover guides.

[0075] The sampler, in another configuration of the device, as illustrated in FIG. 2, provides for collection of multiple samples in a two-dimensional array of individual sample cells. The sampler has provision for automated reading of sample data using bar-coding [2-1] to ensure correct identification of tissue sample and its corresponding DNA sample following purification of the DNA by the automated DNA preparation device. The sampler has a retractable sliding base [2-2] and lid [2-3] for convenient transport of the tissue and automated transfer of individual tissue samples into the tissue mill as shown in FIG. 3.

[0076] The additional parts of the mill that provide for the automated delivery of plant tissue from the tissue sampler into the mill:

- Figure 3-1 main housing;
- Figure 3-2 front cover plate;
- Figure 3-5 plunger;
- Figure 3-6 pinion gear - sample holder;
- Figure 3-8 linear sample holder;
- Figure 3-10 cover strips [lid and base].

[0077] The sample holder is located by a visual cue located on the holder body within the main housing [3-1]. Automated retraction of the lid and base slides [3-10] allow motorised entry of the plunger [3-5] to push the plant tissue at a measured speed into the mill entry port. An o-ring on the plunger prevents buffer leakage. Following compete processing of the sample in the mill, the plunger is retracted and the sample holder is progressed by the pinion gear to allow entry of the subsequent tissue sample into the mill.

EXAMPLE 2

Tissue mill

[0078] The tissue mill, as illustrated in FIGS 4 and 4A, lessens plant cell disruption and automation provides the liberation of high yields of "high quality" cell nuclei.

[0079] The parts of the automated Mill that provide for the homogenisation of plant tissue are shown in Figure 4 in one embodiment, being the numbered parts:

- Figure 4-1 mounting block;
- Figure 4-4 leadscrew -position adjustment;
- Figure 4-5 main housing -housing stator & rotor;
- Figure 4-7 baseplate for assembly;
- Figure 4-8 guide shafts;
- Figure 4-9 main spindle shaft;
- Figure 4-10 helix-feed of plant tissue into stator -rotor milling area;
- Figure 4-11 rotating part of the mill [rotor];
- Figure 4-12 stationary part of the mill [stator];
- Figure 4-13 actuator housing;
- Figure 4-17 main spindle motor.

[0080] The operation of the Mill being: the clearance between the rotor [4-11] and stator [4-12] is automatically adjustable and processor controlled via leadscrew [4-6] and a servo motor [4-13] along the guide shafts [4-8] to locate the entire main motor [4-17], rotor [4-11] helix-feed [4-10] and spindle shaft [4-9] at the appropriate clearance. The rotor is actuated by the main spindle motor at a controlled speed of rotation. Homogenisation buffer is fed into the upper body of the mill above the entry port for the plant tissue to ensure continuous flushing of the mill during operation. The tissue homogenate exits at the bottom of the mill into a mixing chamber [Figure 7] described below. Following completion of the sample commination, the clearance between the rotor and stator is increased markedly by controlled

descent of the rotor, drive shaft and main spindle motor along the guide shafts [4-8], accompanied by a flushing of the mill parts with a washing solution which leads to waste.

[0081] It is apparent that a motorized, high torque, low shear, precision clearance screw mill can provide several advantages over the high-speed homogenizers and manual homogenizing methods. The low speed homogenization process avoids extensive organelle disruption and attendant DNA shear and the continuous provision of homogenization buffer solution during milling provides protection for the DNA which remains within the cell nucleus. The conical helical screw mill and precision milling surfaces designs of the mill rotor and stator ensure high efficiency delivery of tissue to the cutting surfaces and provide for through-flow of both partially homogenized plant tissue towards precision milling surfaces. They further provide for through-flow of fully homogenized tissue to outlet the mill and the adjustable, precision clearance between rotor and stator provides for a high degree of cell breakage from both soft and tough woody tissues.

[0082] An area of high precision clearance between the rotor (FIG 5) and the stator (FIG 6) ensures that tough plant cell walls of woody tissues are effectively broken resulting in the release of nuclei free from the cell debris in high yields. In particularly, the design of the cutting teeth and associated conduits allows for the flow of tissue homogenate from the posterior inlet to the outlet face at the anterior of the rotor, via the mill rotor.

[0083] The working parts of mill termed the Rotor and Stator shown previously as part 4-11 and part 4-12 respectively provide for the homogenisation of plant tissue within the automated mill. These rotor and stator in one embodiment are shown in greater detail in Figure 5 and Figure 6 respectively, having the particular features:

- Figure 5 Rotor cone;
- Figure 5-1 fine clearance surface. Clearance between the rotor and stator adjusted to a defined particle size.
- Figure 5-2 attachment to the main drive spindle;

- The rotor cone tooth depth decreases across the surface of the rotor towards the wide end. Teeth are helically aligned across the surface of the rotor.
- The distance between the teeth increase towards the wide end of the rotor.
- The narrow end face of the rotor attaches to the helix-feed worm [part 4-10].
- Figure 6 Stator cone;
- 1.5mm clearance at the start [narrow] end. Clearance tapers out across length of the stator.
- Fine clearance at the wide-end for approximately 1-2 mm. End acts like a grinding surface.
- Sharp tooth edges (on inner diameter) promote tissue cutting;
- Root of tooth filleted to avoid clogging.

[0084] The design of the teeth and conduits are helical across the expanding conical cutting surface of the rotor and the depth of the conduits decrease and increase in width towards the anterior [wide end] of the mill, ensuring a constant volume clearance between rotor and stator across the working surfaces (Figures 5). Similarly, the stator has sharp teeth which are aligned longitudinally to promote cutting of the tissue as the plant material is worked past the stator teeth during rotation of the rotor. Further, the root of the teeth are filleted to avoid tissue clogging and the distance between teeth increases along the length of the conical stator towards the wide end.

[0085] The clearance between the rotor and stator is varied across the length of the mill, such that following helix-feed worm [4-10], delivery of tissue into the posterior [narrow end] of the mill there is sufficient clearance for the partially intact plant tissues. The forward movement of the tissue towards the anterior [wide end] of the mill and corresponding commination of the tissue occurs across the mill working surfaces in which the clearance between the rotor and stator diminishes until a set minimum clearance between rotor and stator is achieved. This minimum clearance is then constant across the remaining portion of the *mill* working surfaces, providing for fine milling of the plant tissues prior to exit from the

mill. The volume available for occupation by the plant tissue is relatively constant across the length of the mill, provided by both the increasing surface area of the conical rotor and stator and the increasing width in the spacing between the helically aligned teeth on the rotor and longitudinally aligned teeth on the stator.

[0086] The automated features of the mill include, anterior feed of homogenization buffer contiguous with mill operation to ensure that plant tissue is continuously surrounded by protective buffer during all stages of homogenization, automated and controlled setting of clearance between cutting surfaces on the rotor and stator, and a series of ring spray outlets to provide for a high flow of washing solution to all working surfaces of the mill during an automated cleaning process. Further the mill has precise temperature control and an automatic system for the delivery of plant leaf tissue into the homogenization chamber.

[0087] In use, the tissue sample is fed into the rotating helix, which chops the sample into maximum 2mm long strips using the lower edge of the sample feed in hole as a cutting edge. Smooth resistance free faces prevent clogging. The sample moves into the rotor and stator as the helix shares the same tooth depth. The sample then flows along the tooth of the rotor. As each tooth from the stator passes over the sample, it has the freedom to move, rotate and is chopped into small pieces as the two cutting edges pass. All teeth have radii in the root of the teeth to prevent clogging. As both rotor and stator teeth taper out to 0mm the sample gets smaller and smaller as it reaches the output. The last 1.5 to 2.0mm of the stator is a fine grinding area.

[0088] The surface finish and titanium nitride ceramic coating assists in clog free milling. Any debris, which is left behind after mill, is easily flushed away.

EXAMPLE 3

Homogenization Buffer

[0089] Any suitable buffer solution in which the plant tissue is homogenized that is known to those practised in the art may be used. Typically, the buffer solution includes agents to

protect the integrity of nuclei, to protect the DNA integrity by inhibiting the activity of nuclease enzymes and to inhibit oxidation processes that promote the polymerization of reactive phenolic compounds. The buffer includes additives such as soluble polyvinylpyrrilidone (PVP), dithiothreitol and ethylenediamine-tetraacetic acid (EDTA) and a non-ionic detergent, such as Triton, Nonidet or Tween, to facilitate the dispersal of plant cell membranes and to promote the solubilization of proteins, polysaccharides and phenolic compounds. However, it is to be understood that the foregoing additive compounds are not exclusive, and that additional or alternative compounds could be included or substituted for the forgoing compounds. For isolation of cell nuclei with high quality DNA from a variety of plant species, the preferred buffer is mildly alkaline, in the range pH 8.0-9.5, in which DNA is stable and the potential DNA-nicking activity of nucleases is inhibited.

EXAMPLE 4

Post-homogenisation mixing chamber

[0090] The homogenised tissue slurry emerging from the mill may not be fully dispersed and the tissue may not be uniformly distributed throughout the homogenisation buffer. The uniform distribution of plant tissue homogenate and the homogenisation buffer is facilitated by the provision of a mixing chamber which, in one configuration of the device, facilities for the agitation of the slurry (Figure 7). The outflow from the chamber is prevented by an automated sliding valve and provision is made for the filtration of the tissue slurry through a coarse filter to remove larger particles of unbroken tissue prior to the subsequent steps involved in purification of the liberated cell nuclei.

[0091] The parts of the "Mixing chamber" that provide for the automated mixing of the homogenised plant tissue slurry are shown in Figure 7 in one embodiment, being the numbered parts:

- Figure 7-1 peristaltic mixing disc;
- Figure 7-2 outlet tube;

- Figure 7-3 lower casing;
- Figure 7-4 middle casing;
- Figure 7-5 upper casing;
- Figure 7-6 sealing surface;
- Figure 7-7 lower cone;
- Figure 7-8 tube to waste;
- Figure 7-9 collection chamber;
- Figure 7-10 attachment to bottom of mill block;
- Figure 7-11 3-position valve;
- Figure 7-13 outlet tube for tissue slurry;
- Figure 7-14 cleaning liquid inlet.

[0092] A conical attachment [7-7] to the anterior of the mill rotor provides for the collection of emerging homogenate into the centre of the mixing chamber. Agitation of a buffer reservoir by a peristaltic action [7-1] or allow a gentle mixing and distribution of tissue slurry and any residual buffer. Following mixing of the slurry and release of nuclei from plant debris, the homogenate can be filtered coarsely through a mesh filter positioned between the middle casing [7-4] and the lower casing [7-3]. The filtered slurry is then run off [7-13] to the subsequent mixer, flow-centrifuge processing step described below.

EXAMPLE 5

Phase purification of plant nuclei.

[0093] The two-phase buffer solution in which plant tissue homogenate is mixed and contains the polymers dextran and polyethylene glycol (PEG) as the immiscible phase forming agents with the preferred buffer being potassium phosphate in the range pH 7.0-8.0. However, it is to be understood that the foregoing phase-forming polymer compounds are not exclusive, and that additional or alternative compounds could be included or substituted for the forgoing compounds, including such alternative aqueous phase forming systems as Ficol:Dextran,

Uncon:Dextran, PVP/PEG:Dextran, PVP:PEG or Salt:PEG, or some combination of an immiscible polymer with a substituted-Dextran, or a substituted-PEG polymer with Dextran.

[0094]Further, the two-phase buffer solution contains agents to protect the integrity of plant cell nuclei, to protect DNA integrity by inhibiting the activity of nuclease enzymes and to inhibit oxidation processes that promote the polymerization of reactive phenolic compounds. The two-phase buffer includes additives such as soluble polyvinylpyrrilidone (PVP), EDTA and dithiothreitol or β-mercaptoethanol and a non-ionic detergent such as Triton or Tween to facilitate the dispersal of plant cell membranes and to promote the solubilization of proteins, polysaccharides and phenolic compounds. It is to be understood that the foregoing additive compounds are not exclusive, and that additional or alternative compounds could be included or substituted for the forgoing compounds.

[0095]An aqueous two-phase system is the medium used to promote the partition of free plant cell nuclei to the interface region that occurs between the two resolved phases. The rapid resolution of the two-phase system and partition of the free nuclei to the interphase region between the phases is achieved by the exertion of centrifugal or acoustical forces on the mixed phase system. The external force is achieved by centrifugation of the phase system, or by transduction of ultra-sound through the phase system mixture.

EXAMPLE 6

Flow centrifuge with automated sample collection during centrifugation

[0096]The flow centrifuge provides for the rapid isolation of plant nuclei from discrete samples of a plant tissue homogenate, with provision for collection of the aqueous-phase partitioned cell nuclei in a mixer, flow centrifuge shown in one embodiment in Figure 8 and in one embodiment in Figure 9 for the disposal of expended phase buffers to waste and for through-flushing and cleaning of the centrifuge and attendant apparatus prior to the processing of the subsequent sample in a continuous cycle of action. Further detail for designs for a mixer, flow centrifuge shown in one embodiment in Figure 10. The centrifuge

rotor has small mixer/agitator vanes at the base of the bowl to provide for complete low speed alternate rotation for mixing of the introduced phase solutions and the plant tissue solution, prior to centrifugal partition of the aqueous phases. Further detail for designs for arm devices for collection of the partitioned solution and for cleaning and washing of the centrifuge rotor are shown in one embodiment in Figure 11. The collecting arm can be inserted and retracted from the centrifuge bowl at appropriate times. In addition, the arm devices may be employed for mixing of the introduced phase solutions and the plant tissue solution, prior to centrifugal partition of the aqueous phases.

[0097] The parts of the "mixer flow centrifuge" that provide for the automated mixing of phase solution and tissue slurry, and for centrifugation of plant tissue slurry and phase buffers are shown in one embodiment in Figure 8, being the numbered parts:

- Figure 8-1 drive end [x-table];
- Figure 8-3 free end [x-table];
- Figure 8-4 guide shaft;
- Figure 8-5 carrier [outlet tube];
- Figure 8-7 position motor;
- Figure 8-10 centrifuge base;
- Figure 8-12 centrifuge rotor;
- Figure 8-13 drive motor.

[0098] The centrifuge bowl [8-12] is open topped and has several designs for the purpose of accentuated linear separation between the upper and lower phase solutions and for removal of residual tissue debris. The partitioned phase solutions are collected by a collecting arm which is lowered into the surface of the phase solution during operation of the centrifuge [Figure 9]. The collecting arm may include a finer collection needle. The phase solutions are then actively scanned by a video camera and the resolved phase fractions may be identified, allowing a processor controlled, active collection of defined phase fractions by the collecting arm and a motorized pump. Alternatively, the phase solutions may be actively collected by a

motorised pump and the fraction may be monitored spectrophotometrically during collection. The position of the collection arm is controlled by a position motor [8-7] which actuates movement of the collection arm along guide shaft [8-4]. The collecting arm can be inserted and retracted from the centrifuge bowl at appropriate times. The collecting arm has in addition a washing device with spray nozzles to clean the surface of the centrifuge rotor before introduction of the subsequent tissue sample [Figure 11].

[0099] Referring in particular to FIG. 9, the collection arm [9-1] may include a finer collection needle [9-2]. The collection arm may be moved vertically [9-3] or may be rotated [9-4] in order to insert into the centrifuge chamber to collect the fractions. The resolved phase mixture may be actively scanned by a video camera and the resolved phase fractions may be identified, allowing active processor controlled collection of defined fractions by the collecting arm and a motorised pump. The collected fraction may be pumped to a collector of fractions or alternatively to be analysed for detection of the desired fraction [9-5]. The rotor [9-6] may also include a more narrow column [9-7] for better resolution of the fractions.

[0100] The invention is thus designed to function for batch processing of samples in a continuous process cycle. In one preferred configuration, the automated flow centrifuge includes a planar collection reservoir for which motorised automated collecting arm, comprising collection tubing, pumps, valves and optical detection devices provide for the serial introduction of phase systems into a separation environment and for automated collection of the resolved phase fractions from the flow centrifuge during continuous operation of the centrifuge at speed of rotation commensurate with phase resolution.

[0101] Further, in one configuration of the device, the centrifuge rotor is provided with movement of the collection arm into the bulk of the mixture to provide for a turbulent mixing action to solutions added to the rotor and during low speed rotations [9-1]. During automated, continuous operation of the centrifuge, the buffer solutions and buffers containing plant material, phase buffers or other appropriate solutions or additives may be added to the mixing chamber and the phase buffer and other ingredients gently mixed

together by the turbulent action of the collection arm [9-1] at low speed. The withdrawal of the collection arm and an increase in the speed of rotation in the centrifuge mode causes the solutions and materials to migrate along the walls of the centrifuge under the influence of the applied centrifugal field until the solutions accumulate in a planar annular collection reservoir at the radial extremity of the rotor.

[0102] Further, in one configuration of the device, the centrifuge rotor is provided with a plurality of axial mixing vanes in a collection chamber at the base of rotor to provide for an impeller mixing action to solutions added to the rotor and during low speed rotations in alternate directions [10-1 & 10-2]. During automated, continuous operation of the centrifuge, the buffer solutions and buffers containing plant material, phase buffers or other appropriate solutions or additives may be added to the mixing chamber and the phase buffer and other ingredients gently mixed together by the impeller action of the mixing vanes or agitator [10-3] at low speed. An increase in the speed of rotation in the centrifuge mode causes the solutions and materials to migrate along the walls of the centrifuge under the influence of the applied centrifugal field until the solutions accumulate in a planar annular collection reservoir at the radial extremity of the rotor.

[0103] Further, an automated retractable siphon arm can be inserted vertically into the operating rotor through the open top of the rotor, and by a rotational movement bring the siphon into contact with the surface of the phase solutions during centrifugation. The speed of rotation of the centrifuge may be reduced to an angular velocity commensurate with the retention of the phase solutions in the annular collection chamber. Active pumps within the aforesaid arm allow collection of the buffer solution via the siphon probe (Figure 11). Optical sensing devices placed it the path of the siphoned solution are used to detect the different fractions of the phase solution and processor controlled valves allow for routing of particular sample fractions for preservation or to waste.

[0104] Referring in particular to FIG. 11, the centrifuge [11-1] rotates in the direction of the arrow [11-2]. Probe siphons [11-3] and [11-4] are independently movable into the rotor

cavity [11-5] vertically and rotation wise. One probe may be a fine probe for sample collection, whereas the other probe may be wider to assist debris removal. The centrifuge may also include a washing spray head [11-6].

[0105] FIG. 11B depicts the probes in more detail. The probe includes a siphon [11B-1] to collects a fraction to be pumped out [11B-2] for collection or for monitoring. The probe may also include a wash conduit [11B-3] to flush out [11B-4] the centrifuge. As illustrated in FIG. 11A, the washing spray head includes a conduit [11A-1] for the wash to spray out the head [11A-2].

EXAMPLE 7

Phase resolution using Ultrasound

[0106] In one configuration of the device (Figure 12) use is made of an active piezoelectric force transducer to apply mechanical oscillation to the aqueous two-phase system at a resonance frequency of approximately 1MHz in an appropriate ultrasound reflective vessel to accelerate the resolution of the phase system and provide for the migration of the free cell nuclei to the interface region between the two phase polymers. The piezoelectric force transducer is provided with electrodes and is activated by appropriate RF amplifier. Under the influence of ultrasound Lamb-wave forces many nuclei aggregate together into large particles over small distances which migrate to the interface region that occur at the standing minima caused by the superimposition of active and reflected ultrasonic waves. Alternatively, two piezoelectric force transducers may be provided and driven at different frequencies to provide for the directed movement of the nuclei particles towards one of the transducers. Subsequent to the resolution of the phase system, the interface region can be collected from the settled phases by pumping the resolved solutions out of the separation chamber and by optical detection of the nuclear material at the interface the nuclei-rich fraction can be collected separated from the bulk of the upper and lower phase solutions. The partition of free plant cell nuclei in the phase system exposed to ultrasonic standing

waves occurs without any damage or alteration to the length of the DNA contained within the nuclei aggregates and thus provides a rapid and convenient method for the concentration and partial purification of plant cell nuclei from a crude filtered homogenate of plant tissues. Particularly, the application of ultrasound has purpose particularly in highly minaturized DNA isolation systems.

[0107] Referring in particularly to FIG. 12, piezoceramic force transducers [12-1] and [12-2] or alternatively piezoceramic force transducer [12-1] and reflective acoustic mirror material (plastic or glass) [12-2] are located on the ends of a thin walled resonance tube [12-3]. Valves [12-4] and [12-5] are the inlet and outlet of the phase mixture and for the collection of the phase resolved nuclei. The whole assembly in enclosed in a carrier module [12-6] to facilitate rotation about the central axis [12-7]. The tube may be vertical for loading and empty and horizontal for ultrasonic phase resolution.

EXAMPLE 8

Nuclear Lysis

[0108] The solubilization of DNA is promoted by the addition of a buffer solution containing a broad-spectrum, heat-stable proteinase enzyme, such as proteinase K [from *Tritarachium album*, EC 3.4.21.64] and a non-ionic or ionic detergent to the partially purified plant cell nuclei to achieve the digestion of histones and nuclear membrane-associated proteins and result in the solubilization of free DNA into the buffer solution. The protease-mediated digestion is performed at an elevated temperature, in the range of 55-65°C. However, it is to be understood that the foregoing proteinase enzyme and additive compounds are not exclusive, and that additional or other proteinase enzymes or alternative compounds could be included or substituted for the foregoing compounds.

EXAMPLE 9

DNA and RNA

[0109] An aqueous two-phase system is the preferred medium used to promote the partition of free plant DNA into the dextran polymer phase following the resolution of the aqueous phase mixture. The rapid resolution of the two phase system and partition of the free DNA is achieved by the exertion of mechanical forces on the mixed phase system. Without being bound by theory, the external force is achieved by centrifugation of the phase system as described above, or by transduction of ultra-sound through the phase system as described above.

[0110] In one embodiment a mixer, flow centrifuge is shown in Figure 10. The centrifuge rotor has small mixer/agitator vanes at the base of the bowl to provide for complete low speed alternate rotation for mixing of the introduced phase solutions and the plant tissue solution, prior to centrifugal partition of the aqueous phases. The partitioned phase solutions would be centrifuged in the wide chambered centrifuge bowl [10-2] and the upper phase, lower phase and interface material collected by the siphon arms described above and shown in one embodiment in Figure 11. Free DNA efficiently partitions into the lower phase and is collected for further processing to purity.

EXAMPLE 10

Concentration and purification of DNA by ElectroRetention

[0111] Free plant DNA is concentrated from solution into a solid media by application of an electric field perpendicular to the direction of bulk laminar fluid flow. The solid media severely reduces the electrophoretic migration of the DNA relative to its migration in free solution, allowing the nucleic acid to accumulate. In the present invention the DNA which has partitioned into the dextran polymer phase of an aqueous two-phase system is recovered, by introduction of the phase solution into an electrical flow cell in which semi-

porous membranes [e.g cellophane dialysis membrane] separate the DNA solution from both the anodic and cathodic electrodes, and the cell contains a thin layer of an appropriate DNA sieving medium. In the present invention, in one configuration of the device shown in Figure 13, the electrophoretic flow cell is provided with several compartments that are electropermeable for the flow of ionic species, but which maybe relatively impermeable to high molecular weight polymers or matrix particles. The electrophoresis buffer surrounding the cathode and anode electrodes is bound within chambers connecting to the exchange compartments by a semi-permeable membrane such as cellophane or dialysis membrane. A high flow rate for electrophoresis buffer exchange within the electrode chambers reduces Joule heating and prevents acidification and alkalisation of the exchange buffers within the connecting exchange compartments. The relatively dilute free DNA within the nuclear digestion solution enters the "phase compartment" of the flow cell (Figure 13). This solution may be either a phase buffer fraction, or another form of a low-ionic strength buffer into which the plant DNA has been liberated by action of the protease in a previous step.

[0112] In one embodiment, the sieving medium within the electro-retention compartment is not solid gel, but is an entangled form of solution [Cheng et al. 1994, Analytical Chemistry 66:4210-4214; Righetti 1995, Journal of Chromatography 698:3-17; Chiari and Righetti 1995, Electrophoresis 16:1815-1829], such as liquid agarose polymer, or soluble cellulose polymers such as hydroxypropyl methyl cellulose and hydroxyethyl cellulose, linear polyacrylamide, linear N-acryloylaminoethoxyethanol, or very high molecular weight dextran. In order to enhance the retention of DNA by the sieving matrices based on linear polyacrylamide or linear N-acryloylaminoethoxyethanol, DNA affinity ligands such as ethidium, phenyl neutral red, or fuchsin might be copolymerized in conjunction with the polyacrylamides. The use of an entangled solution matrix facilitates the replacement of expended sieving medium from the electrophoresis flow chamber and its replacement by the introduction of fresh sieving medium. The sieving medium is located on the cathodic side of the flow-cell. The interface between the bulk fluid introduced into the flow cell and the

sieving medium is bounded by a macroporous membrane that permits free electro-migration of DNA molecules between the sieving medium and the flow cell, and yet prevents easy migration by diffusion of the liquid sieving medium back into the laminar flow cell.

[0113]Without being bound by theory [Slater et al. 1996, Methods in Enzymology 270:272-295; Kléparník et al. 1997, Journal of Chromatography A 772:243-253], the application of a direct current electrical field induces the DNA to migrate towards the anode and is thus driven from the isolation buffer into the sieving matrix medium. The size selectivity of the sieving gel for migration of DNA provides for a selective retention of large DNA molecules within the gel, while smaller, partially degraded DNA molecules or RNA are not retained by the sieving matrix and pass through it. Residual proteins are for the most part of opposite net charge from DNA or possess much fewer charge per unit length and thus either migrate towards the cathode or migrate slowly towards the anode. Neutral molecules and polymers such as polysaccharides and polyphenolic compounds tend to remain within the buffer solution and do not migrate under the influence of the applied electric field. Adequate flow exchange of the DNA containing solution through the flow-cell provides for the replacement of DNA-depleted solution with fresh solution, while also removing contaminant molecular species from the flow-cell. Adequate exchange of electrophoresis buffer in chambers immediately surrounding the electrodes and bounded by the dialysis membranes provides for maintenance of the pH of the DNA solution and sieving matrix during the electrophoretic transfer process.

[0114]Following completion of the electroretention stage, the buffer in the flow-cell is replaced and residual "crude" DNA solution is flushed out. The polarity of the electrodes may be then reversed and by application of appropriate pulsed-electric fields the long DNA is released back into the solution contained within the flow-cell. Alternatively the sieving polymer, containing the retained long DNA, may be retrieved from within the retention compartment of the electrochromatography cell. DNA may then be recovered directly from the sieving medium, or stored within the medium for further use.

[0115] Electro-Affinity retention matrices based on the foregoing polyacrylamide DNA-affinity ligands and silica-based affinity ligands require additional treatment in order to release the retained DNA. DNA may be released by alteration of the ionic environment by an alkali salt of the halide series such as NaI, KI or KCl following recovery of the matrix from the electroretention cell.

EXAMPLE 11

Concentration of pure DNA by Affinity Resin

[0116] An alternative method for the purification of the plant DNA from free solution is achieved in which the dextran phase solution is introduced into a flow-cell in which a solid DNA specific dye-affinity matrix is located. The affinity matrix may selectively bind DNA and retain it from the bulk solution, allowing that fresh DNA containing solution may replace DNA-depleted solution within the flow cell until a saturation of dye-binding sites is achieved.

[0117] The specificity an affinity-dye may be utilized to direct the DNA binding but have attached either ethidium, Hoechst 33258, resorcinol or via primary amine, hydroxyl, or quaternary amine functions respectively to the hydroxyl groups on the surface of fine polyacrylamide particles or fine silica dioxide particles using a glutaraldehyde linker moiety. Glutaraldehyde is a well known crosslinking agent which has been used extensively for the covalent attachment of both proteins and nucleic acids to solid polymers and surfaces such as polyacrylamide, polyethylene, nylon and nitrocellulose [Komissarenko and Avrameas, (1978) Ukr Biokhim Zh 50:500-511; Timofeev et al. (1996) Nucleic Acids Research 24:3142-3148; Sano et al. (1993) Biomaterials 14:817-822; Panikkar et al. (1997) Artificial Cells and Blood Substituent Immobilization Biotechnology 25:541-550; Prabhune and SivaRaman (1991) Applied Biochemistry and Biotechnology 30:265-272; Kelleher and Juliano (1984) Analytical Biochemistry 136:470-475; Guesdon and Avrameas (1976) Journal of Immunological Methods 11:129-133; Karey and Sirbasku (1989) Analytical Biochemistry 178:255-259], however DNA-binding dyes are not conventionally attached to solid matrixes

by this procedure. Alternatively, an ethidium-acrylamide affinity resin for DNA binding may be utilized in which the ethidium is attached to polymerizing acrylamide or bisacrylamide polymer to form an affinity surface, or a linear polyacrylamide liquid gel [Vacek et al. 1982 Analytical Biochemistry 124:414-420]. The free DNA can be bound to the affinity matrix material either by the passage of the solution over an affinity surface or over finely-divided affinity particles. Alternatively, finely divided affinity particles may be placed within the retention chamber of the electrophoresis-flow cell (Figure 13) and the DNA electro-migrated from the "phase compartment" into the "retention buffer" compartment which is the particles of solid affinity matrix reside. Alternatively, finely-divided affinity particles may be placed within the retention chamber of the chromatographic cell (Figure 13) and the DNA allowed to move from the "phase compartment" into the "retention compartment" which is the particles of solid affinity matrix reside by fluid flow.

[0118] The use of electrophoretic transfer of DNA onto an DNA-affinity matrix is that proteins, polysaccharides and other biomolecules that possess an opposite net charge to DNA, or possess a net neutral charge will either electromigrate towards the cathode in the opposite direction to the migration of the DNA, or will not electro-migrate respectively, and thus will reduce contact of such contaminant biomolecules with the affinity matrix. The application of electrophoresis in addition to affinity retention of DNA is superior to the simple use of affinity chromatography to effect contact between DNA and the affinity matrix, because in the former procedure an electrophoretic selection is imposed on the access of biomolecules to the affinity matrix, whereas in the latter chromatographic procedure all biomolecules contact the affinity matrix.

[0119] The use of chromatographic transfer of DNA onto a DNA-affinity matrix is that proteins, polysaccharides and other biomolecules that do not interact specifically with the DNA-affinity ligand will not bind to the ligand, and thus will reduce contact of such contaminant biomolecules with the affinity matrix. Although in the chromatographic procedure all biomolecules contact the affinity matrix, the application of fluid flow with a low

ionic strength buffer through the chromatographic cell, is both rapid and sufficient to remove all contaminant biomolecules, and it is superior to the use of electrophoretic selection because of the speedy removal of contaminant biomolecules from the affinity resin and bound DNA, and additionally because the use of electric fields is not required.

[0120] Subsequent to the transfer of DNA onto the affinity matrix, the solid particles may then be recovered from the electrophoresis-flow cell by an active flushing with low-ionic strength buffer and the particles retained in an end-stop filter. Alternatively, the solid affinity matrix maybe contained within the "retention buffer compartment" (Figure 13) and by an operation of valves achieve a buffer exchange such that the wash solution may pass through the retained affinity matrix to achieve removal of non-specifically bound biomolecules.

[0121] Further, any suitable washing buffer and procedure may be used that effectively elutes non-specifically bound residual nuclear materials from the binding matrix, while continuing to retain bound DNA. Furthermore, any effective eluant of both long and short DNA molecules from the affinity-dye matrix may be used which allows for the quantitative recovery of long pure genomic DNA. It is preferred that these gentle procedures that avoid excessive shear to the DNA into a solution.

[0122] Referring in particular to FIG. 13, the electro flow cell [13-1] may include and buffer inlet [13-2] and buffer outlet [13-3]. The buffer may be a low ionic strength electrophoresis buffer to prevent acidification and alkalization of the DNA phase buffer [13-4] and retention buffer [13-5]. The electrophoresis buffer surrounds the cathode [13-6] and anode electrodes [13-7] which is bound within chambers connecting to the exchange compartments by a semi-permeable membrane such as cellophane or dialysis membrane [13-8]. An electropermeable and DNA permeable membrane [13-9] separates the phase buffer and retention buffer. The DNA electrophoresis from the phase buffer across the permeable barrier into the retention buffer as indicated by the direction of the arrows [13-10] in accordance with the applied electric field [13-11].

EXAMPLE 12

Vacuum Recovery of precipitated DNA

[0123] Pure DNA is routinely recovered from aqueous salt solutions by the addition of adequate concentrations of short-chain alcohols that initiate the rapid denaturation, the aggregation of massed DNA molecules and the precipitation of the denatured DNA as a semi-solid material. The recovery of the DNA can be aided by the removal of excess alcoholic solution through a ultra-fine pore membrane facilitated by the vacuum filtration of the solution. DNA may be collected onto commercially available ultra-fine pore membranes in a 96-well array. The precipitated DNA will be washed with ethanolic solution at 0-4°C to remove all residual salt-containing buffer, prior to dissolution of the DNA in pure water and finally distribution into 96-well array storage vessels.

EXAMPLE 13

Flow sheet for Automated Preparation of Plant DNA

[0124] The plant tissue mill can deliver high purity long DNA from both soft tissue and tough (woody) plant tissues. The mill and attendant devices for the purification of DNA resides broadly in a method of purifying long genomic DNA from "high quality" plant cell nuclei following the mechanical disruption of plant cells by the use of a series of aqueous two-phase partition procedures, affinity chromatographic capture of the DNA, salt elution of DNA and recovery of pure DNA following ethanolic precipitation and vacuum filtration onto 96-well micropore filter arrays. The steps below describe the preferred devices and procedures for the purification of plant DNA. The steps are also described in Figure 14.

[0125] The operational steps involve the following elements:

Field-collected plant tissue samples

i collection of plant tissue in a array field sampler for ease of collecting individual samples into an ordered set of sample cells and to facilitate data logging.

ii The tissue is kept cool until brought to the machine.

Sample entry to the mill

i The tissue sampler is placed on a refrigerated store on the mill until tissue use.

ii The mill has devices for the automated delivery of individual plant tissue samples into the mill for homogenisation.

The Milling of the tissue

i Plant cell nuclei are being released into a protective buffer by continuous delivery of isotonic buffer into the upper mill screw during all milling procedures.

ii Release of plant cell nuclei occurs using low-shear milling equipment and methods of wet milling. The buffer is delivered at ice temperature and contains nuclease inhibitors, antioxidants and other additives.

iii The mill has processor controlled clearance between stator and rotor as well as automatic speed control, flush control and process control.

iv The mill and all devices are flushed free of any residual homogenate.

Homogenate mixer and coarse filter

i The homogenate is then thoroughly mixed at low speed with residual homogenisation buffer to provide complete distribution of tissue throughout the buffer and to promote release of nuclei from the broken cell debris.

ii Coarse cell debris is removed by coarse filtration

Nuclei enrichment in an aqueous two phase buffer in Mixer-Flow Centrifuge 1

i The tissue filtrate is mixed with an aqueous two phase buffer by impeller action in a mixer-flow centrifuge at low speed agitation.

ii The centrifuge speed is increased, the phase mixture migrates to the inner rim partition area of the centrifuge and separates into:- an upper phase rich in protein and free membranes. an interface region rich in nuclei and some cell debris, and a lower phase rich in larger cell debris.

iii The centrifuge speed is reduced to a speed at which the resolved phase suspensions remain in the inner rim region of the centrifuge rotor.

iv An phase collection tube is lowered into the revolving centrifuge rotor and the collection tip is automatically rotated into the surface of the moving phase system.

v Automatic collection of a nuclei-rich fraction of the resolved phase mixture in which:-

The upper phase system is aspirated and sent to waste

The interface system between the phases is aspirated and this nuclei-rich suspension is collected and delivered to the second mixer-flow centrifuge.

The lower phase which is polysaccharide rich, and collects any residual plant cell fragments is aspirated to waste.

vi The centrifuge and collection tubes are flushed out automatically to remove any residual materials.

Nuclear lysis to release free DNA in Swing-out Centrifuge 2

i The nuclei-rich interface suspension is mixed with an aqueous buffer containing a broad-action protease by turbulent impeller action in a conduit delivering the nuclear mixture into a disposable centrifuge tube held in a metal array assembly holding multiple samples.

ii The protease digests the nuclear membrane proteins and histones at 65 °C to liberate free DNA into solution. The metal assembly aids the thermal transfer and helps to maintain the mixture at 65 °C.

iii The nuclear lysate is centrifuged in a swing-out centrifuge at high speed and the diluted phase mixture separates into an upper solution rich in degraded protein and free DNA, and an deposited region at the bottom of the tube rich in residual nuclear and cell particulate debris.

iv The upper clarified solution containing free-DNA is collected by the automated collection arm and disposible pipette tips. Multiple samples are collected simultaneously.

vi The centrifuge tube and pipette tip attachments are disposed and replaced with new plastic wares before addition of the subsequent sample.

Collection of the DNA bound with the Affinity matrix by End-filtration

i The affinity matrix particles are located on a 20 μm filter mesh in a disposable chromatographic column.

ii Affinity binding of the clarified DNA-rich buffer to a high-specificity DNA-binding matrix by passing the buffer solution through a column of affinity matrix particles in a disposable chromatographic column by fluid flow. The flow of fluid may be aided by light pressure to speed the process.

iii Residual phase buffer or nuclear lysis buffer or digested nuclear proteins is removed by extensive washing of the affinity matrix by a low-ionic strength wash buffer.

Release of pure DNA from the affinity matrix

i Pure DNA is released from the affinity matrix by exposure to salts [e.g. 1M to 3M KI [potassium iodide] in 20 mM Tris HCl, pH 7.8] or by agents that reduce the affinity of the matrix for DNA and cause a quantitative release of DNA into a small volume.

Recovery of the DNA from solution

i DNA is precipitated by alcohol denaturation of DNA and the denatured DNA is collected onto a Millipore Corp. polypropylene deep-well, 96-well array by centrifugation.

ii The precipitated DNA aggregate is washed with ice-cold 70% ethanol solution containing 10 mM ammonium acetate, pH 8.0 to remove residual KI salts.

Alternative Recovery of the DNA from solution

i DNA is precipitated by alcohol denaturation of DNA and the denatured DNA is collected onto a Millipore Corp. 0.22 um PVDF membrane 96-well array by vacuum filtration.

ii The filtered DNA aggregate is washed with ice-cold 70% ethanol solution containing 10 mM ammonium acetate, pH 8.0 to remove residual KI salts

Resuspension of purified genomic DNA in 0.1xTE buffer or Water

i The purified genomic DNA in 0.1xTE buffer [1.0 mM Tris, 0.1 mM EDTA, pH 8.0] to a desired volume and concentration.

ii The dissolved DNA solution is collected from each collection well and re-deposited into 96-well storage vessel tray.

iii A multiple tip sampler probe removes an aliquot for automated determination of DNA concentration by fluorescence emission. This will involve mixing a metered amount of fluorescent dye with the DNA aliquot and sequential transport of the sample mixture into a fluorometer cell.

iv Sample concentration will be calculated automatically and stored electronically.

EXAMPLE 14

[0126]The operational steps for automated preparation of plant DNA involve the following preferred elements:

Preferred Procedures

- Field-collected plant tissue samples
- Sample entry to the mill

- The Milling of the tissue
- Homogenate mixer and coarse filter
- Nuclei enrichment in an aqueous two phase buffer in Mixer-Flow Centrifuge 1
- The interface system between the phases is collected and delivered to the nuclear lysis chamber in Swing-out Centrifuge 2.
- Nuclear lysis is caused by protease digestion
- Nuclear lysis to release free DNA in Swing-out Centrifuge 2
- The upper solution phase containing free-DNA is collected.
- Collection of the Affinity matrix bound with DNA by End-filtration
- The affinity matrix particles are captured on a 20 μm filter mesh.
- Release of pure DNA from the affinity matrix by 1M-3M KI
- Recovery of the DNA from solution
- DNA is precipitated by alcohol denaturation of DNA and a precipitate is collected by centrifugation, or by vacuum filtration.

Resuspension of purified genomic DNA in 0.1xTE buffer or Water

[0127] Sample concentration will be calculated automatically and stored electronically.

Alternative 1#

The steps below describe an alternative operational use of the preferred devices and procedures for the purification of plant DNA. The steps are also described in **Figure 15**.

[0128] Operational steps involve the following elements:-

Same as Preferred

- Field-collected plant tissue samples
- Sample entry to the mill
- The Milling of the tissue
- Homogenate mixer and coarse filter

- Nuclei enrichment in an aqueous two phase buffer in a Mixer-Flow Centrifuge 1
- The interface system between the phases is aspirated and this nuclei-rich suspension is collected and delivered to the second mixer-flow centrifuge.
- Nuclear lysis to release free DNA in a Mixer-Flow Centrifuge 2
- The lower phase solution containing free-DNA is collected

ALTERNATIVE Centrifugal Resolution of an aqueous two-phase polymers and DNA-Affinity Matrix in Mixer-Flow Centrifuge 3

[0129] The interface system with affinity matrix particles between the phases is collected

Same as Preferred

- Collection of the Affinity matrix bound with DNA by End-filtration
- The affinity matrix particles are captured on a 20 μm filter mesh
- Release of pure DNA from the affinity matrix by 1M-3M KI
- Recovery of the DNA from solution
- DNA is precipitated by alcohol denaturationand collected by vacuum filtration.
- Resuspension of purified genomic DNA in 0.1xTE buffer or Water.
- Sample concentration will be calculated automatically and stored electronically.

Alternative Procedure 2#

[0130] The steps below describe an alternative operational use of the preferred devices and procedures for the purification of plant DNA. The steps are also described in **Figure 16**.

Operational steps involve the following elements:-

Same as Preferred

- Field-collected plant tissue samples
- Sample entry to the mill

- The Milling of the tissue
- Homogenate mixer and coarse filter
- Nuclei enrichment in an aqueous two phase buffer in a Mixer-Flow Centrifuge 1
- The interface system between is collected and delivered to the second mixer-flow centrifuge.

ALTERNATIVE Nuclear lysis to release free DNA in a Mixer-Flow Centrifuge 2

[0131] The nuclei-rich interface suspension is digested by protease. A clarified upper solution rich in DNA is freed of pelleted solids and collected.

Same as Preferred

- Collection of the Affinity matrix bound with DNA by End-filtration
- The affinity matrix particles are captured on a 20 μm filter mesh.
- Release of pure DNA from the affinity matrix by 1M-3M KI
- Recovery of the DNA from solution
- DNA is precipitated by alcohol denaturation and collected by vacuum filtration.
- Resuspension of purified genomic DNA in 0.1xTE buffer or Water
- Sample concentration will be calculated automatically and stored electronically.

Alternative 3#

[0132] The steps below describe an alternative operational use of the preferred devices and procedures for the purification of plant DNA. The steps are also described in **Figure 17.**

Operational steps involve the following elements:-

<u>Preferred Procedures</u>

- Field-collected plant tissue samples
- Sample entry to the mill

- The Milling of the tissue
- Homogenate mixer and coarse filter
- Nuclei enrichment in an aqueous two phase buffer in Mixer-Flow Centrifuge 1
- The interface system between the phases is collected and delivered to the second mixer-flow centrifuge.

ALTERNATIVE Nuclear lysis to release free DNA in a Mixer-Flow Centrifuge 2

[0133] The nuclei-rich interface suspension is digested by protease. Supernantant is freed of pelleted solids and a clarified solution rich in DNA is collected.

ALTERNATIVE DNA purification in a Mixer-Flow centrifuge 3

[0134] The clarified DNA solution is bound by solid affinity matrix and mixed with an aqueous two phase buffer. The interface system with affinity matrix particles is collected.

Same as Preferred

- Collection of the Affinity matrix bound with DNA by End-filtration
- The affinity matrix particles are captured on a 20 μm filter mesh.
- Release of pure DNA from the affinity matrix by 1M-3M KI
- Recovery of the DNA from solution by alcohol denaturation and collected by vacuum filtration
- Resuspension of purified genomic DNA in 0.1xTE buffer or Water.

Alternative 4#

[0135] The steps below describe an alternative operational use of the preferred devices and procedures for the purification of plant DNA. The steps are also described in **Figure 18.**

Operational steps involve the following elements:-

Preferred Procedures

- Field-collected plant tissue samples
- Sample entry to the mill
- The Milling of the tissue
- Homogenate mixer and coarse filter
- Nuclei enrichment in an aqueous two phase buffer in Mixer-Flow Centrifuge 1
- The interface system between the phases is collected and delivered to the second mixer-flow centrifuge.
- Nuclear lysis to release free DNA Mixer-Flow Centrifuge 2
- The lower phase solution containing free-DNA is collected.

ALTERNATIVE Electrophoretic Retention of plant DNA

[0136] The DNA-rich lower phase is electrophoresed from an electrophoresis flow cell onto particulate DNA-affinity matrix.

ALTERNATIVE Release of pure DNA from the affinity matrix by 1M-3M KI

[0137] Pure DNA is released from the affinity matrix contained within the flow-cell.

Same as Preferred

- Recovery of the DNA from solution by alcohol denaturation and collected by vacuum filtration.
- Resuspension of purified genomic DNA in 0.1xTE buffer or Water.

EXAMPLE 15

[0138] An example of buffers and methods that may be used in the preparation of nuclear material previously described are as follows:

NIB Buffer: adapted from Liu and Whittier (1994) Nucleic Acids Res. **22**:2168-2169,

Nuclear Isolation Buffer - Basic = NIB Basic **1 litre**

Stock 0.5 M **Sucrose** or **Mannitol**	171 g sucrose,
10 mM Tris, pH 9.5	10 ml of 1M
10 mM EDTA	20 ml of 0.5M
100 mM K Cl	7.455 g

make up to 890 ml AUTOCLAVE

NIB[ALL] BUFFER **for 50 ml**

44.5 ml NIB Basic Buffer

plus	3 ml 25% PVP	= 1.5 %
plus	0.5 ml 1M DTT	= 10 mM
plus	0.75 ml Triton	= 1.5 %

Albertsson Aqueous 2-Phase system [AUTOCLAVED]

2A3-E* phase = 10% Dextran T500; 8% PEG8000; 10 mM EDTA; 20 mM NaPi, pH 7.0

2A3* phase = 10% Dextran T500; 8% PEG8000; 20 mM NaPi, pH 7.0

Albertsson Phases

2xA3 - 10% Dextran T500; 8% PEG8000; 20 mM NaPi. pH 7.0

Albertsson Phases	**2≈3**	**30 ml**	-->	**400 ml**
Dex500	10%,	3.0 g	-->	40 g
PEG8000	8%	2.4 g	-->	32 g
250mM NaPi, pH 7.0	20mM	2.4 ml	-->	32 ml

OPTIONAL

0.5M EDTA	10 mM	0.6 ml	-->	8.0 ml

PVP40	1.0 %	0.3 g	-->	4.0 g
Sucrose [342 g]	0.25M	2.56 g	-->	34.2g
KCl [74.55]	0.3M	0.67 g	-->	8. 90 g

2A3 CENTRIFUGATION METHOD

1. 0.8 ml mixed 2A3, add 0.8 ml Nuclei in isolation buffer and invert/ gently mix 10 sec
2. Form InterFace by gentle centrifugation @ 1,500rpm x 4 min
3. Aspirate away upper then lower phase from InterFace
4. Add 1-2 ml of NIB wash buffer [no detergents or PVP] and wash
5. Re-pellet Interface to bottom of tube @ 4,000 rpm x 6 min
6. Decant was off and resuspend IF pellet in desired medium.

1x TE/PK	**500 ml**	
2.0 mM EDTA	0.3722 g,	or 2.0 ml of 0.5M
10 mM Tris, pH 8.0	0.6055 g,	or 5.0 ml of 1M
Fresh 1 mg/ml Proteinase K	0.5 g	
Can store at -20 °C until use.		

[0139] It will of course be realised that while the above has been given by way of illustrative example of this invention, all such and other modifications and variations thereto as would be apparent to persons skilled in the art are deemed to fall within the broad scope and ambit of this invention as is herein set forth.

EXAMPLE 16

[0140] An example of buffers and methods that may be used in the preparation of affinity matrix previously described are as follows:

Ethidium-Glutaraldehyde-PolyAcrylamide Affinity Resin [EGPA]

1. 4.0 g P4 polyacrylamide beads [fine] into 50 ml TPE at 65 C for 1hr to expand.
2. Activate surface with Glutaraldehyde

   Activate surface with Glutaraldehyde in K Pi 100 mM buffer pH 7.0, stirring continuously at 40-45 °C for several hours: For example A10 = 10% Glutaraldehyde for 4 hr at 50 °C

   Ethanolamine test == light pale yellow = light Glutaraldehyde modification
3. React Ethidium bromide [EtBr] with the activated beads

3a 5.0 ml of 1:1 EGP slurry of activated resin in 100 mM KPi pH 7.0

3b Variable volume 5 µl to 200 µl of 10 mg/ml EtBr at 45 °C or room temp

3c Mix by inversion occasionally over period: 0.5 hr to several hours.

3d Remove any unreacted EtBr by buffer exchange with 100 mM K Pi, pH 7.0

4. Remove any residual Glutaraldehyde binding sites

   After EtBr is bound, react beads with a variable volume of the neutral blocker, Ethanolamine.

EXAMPLE 17

[0141] Ethidium-Acrylamide-P4 PolyAcrylamide Bead-Affinity Matrix [EAP4A]

Polyacrylamide bead affinity gel linked by co-polymerization with acrylamide may be prepared according to the method of: Vacek et al. Anal Biochem. (1982) 124:414-420.

1. TPE Buffer = 36 mM Tris, 30 mM NaH_2PO_4, 1mM EDTA, pH 7.8

1 litre:-

Tris base[121.1]	4.36g	
NaH_2PO_4 anhyd [120.0]	3.60 g	
Na_2 EDTA [372.2]	0.3722 g	Auto pH to 7.65

2. Make a mixture of :-

   6.0 g BioGel P4 suspended/ swollen in **90 ml** of TPE buffer.

   0.316 g Bis Acrylamide [2.05 mmole] in 20 ml TPE

   8.0 mg Ethidium Bromide [EtBr] in 10 ml of TPE

3. Incubate the mixture for 30 min at 60 °C.
4. Chill the mixture to room temperature ~ 20 °C
5. Add 10 ml of TPE containing 0.4 ml TEMED and 0.4 g fresh Ammonium persulphate.
6. Stir the mixture on a magnetic stirrer at low speed for 2 hr at room temperature.
7. Clean-up of Ethidium-Affinity-Acrylamide P4 Gel

   Add to Syringe with glass wool stopper.

   Wash the affinity beads with 300 ml of TPE to remove un-bound reactants.

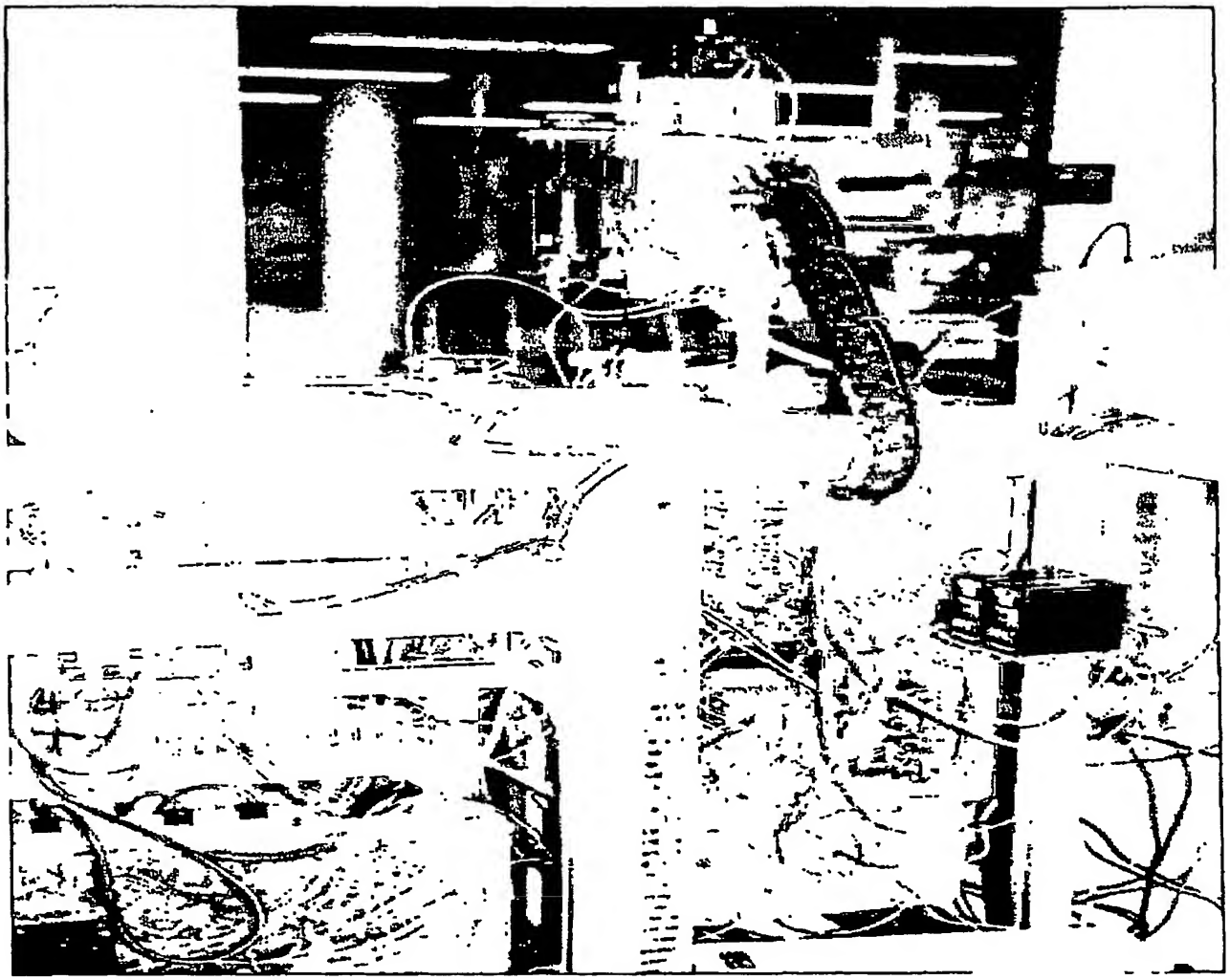
8. Bound EtBr determined by subtraction from wash eluant.

   Total volume. 1 cm path/ 486 nm A = 1.0 = 86 ug EtBr

9. Medium stored in 50 ml TPE at 4 °C.

EXAMPLE 18

[0142]DNA preparation Robot – Side on view of the working prototype version 1



The DNA preparation robot has component parts and application as illustrated in EXAMPLE 26 through to EXAMPLE 31.

Genomic DNA prepared from plant tissue using the DNA preparation robot and methods described herein and applications for the use of such genomic DNA are illustrated in EXAMPLE 32 through to EXAMPLE 37.

EXAMPLE 19

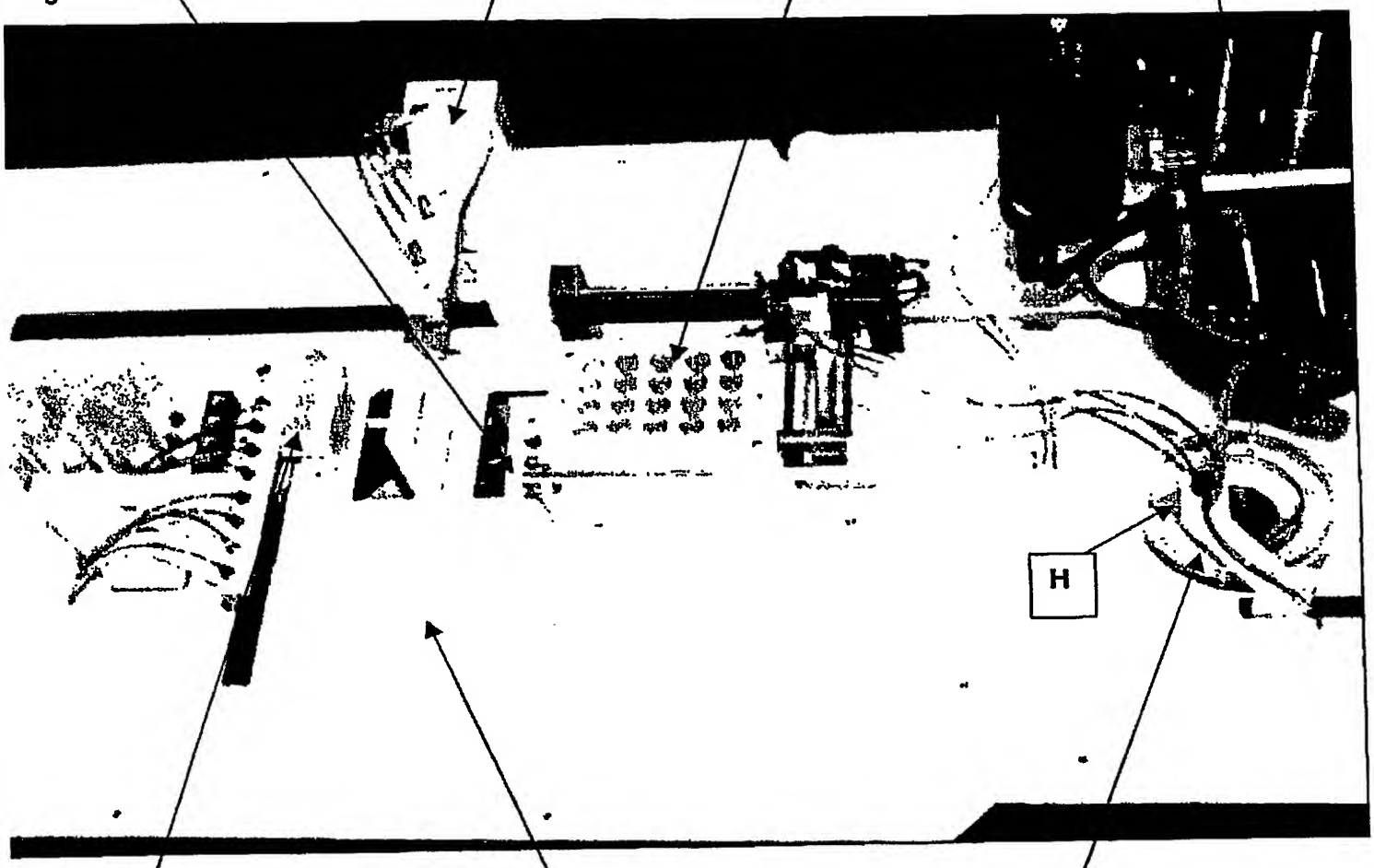
[0143] WORK DECK: Overhead view

Swing-out Centrifuge

Liquid dispenser

Peltier heater

Tissue mill



DNA binding Resin Holders

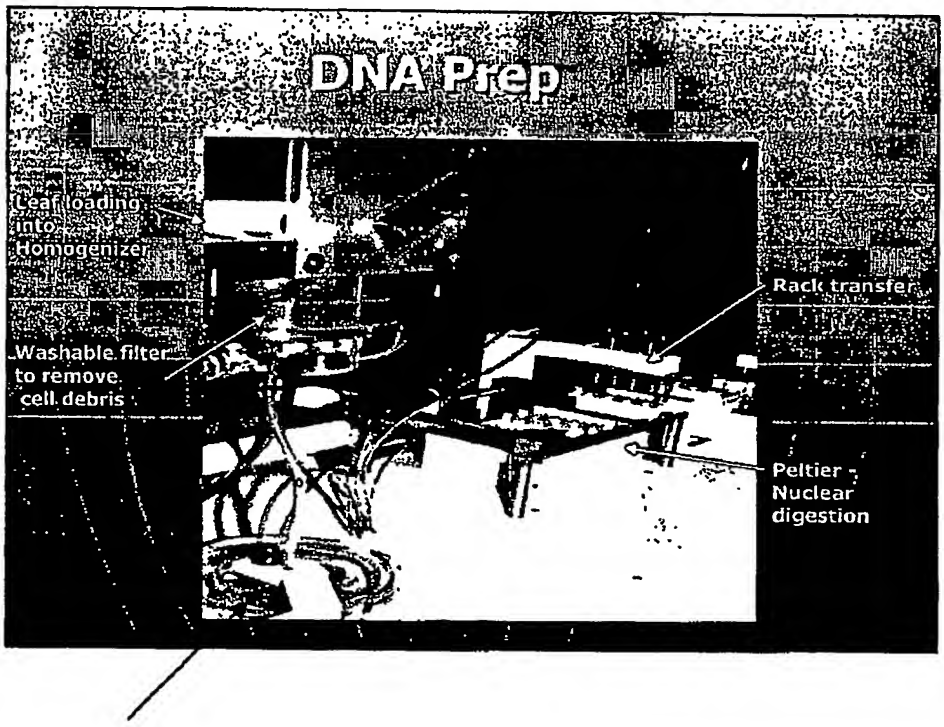
DNA collection wells

Continuous flow annular centrifuge, note the sample collection arm and head [H].

The DNA is prepared in five simple processing steps with tubing linking between each step.

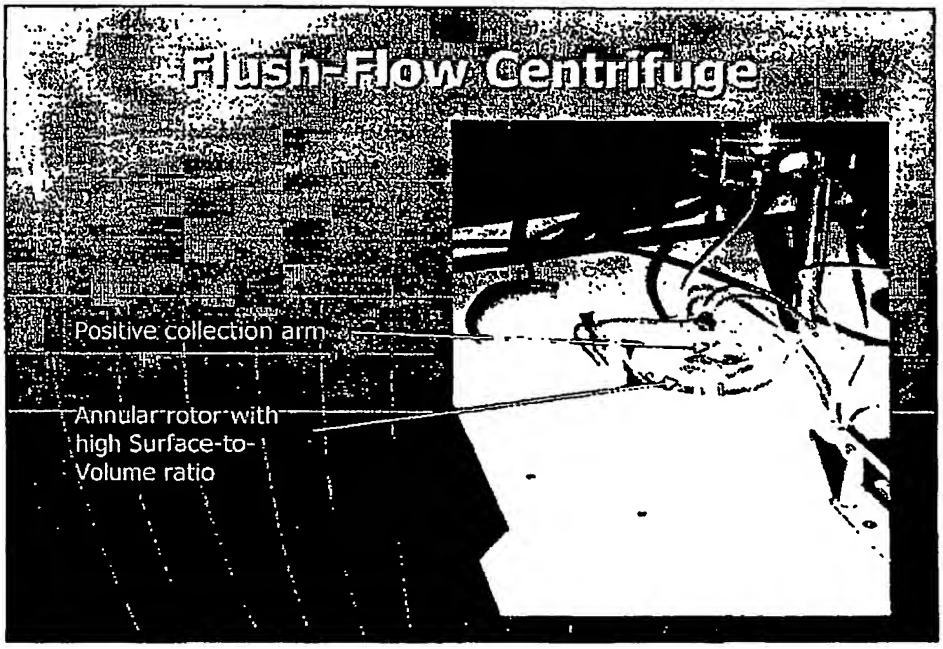
EXAMPLE 20

[0144] Horizontal view of the tissue homogenizer and washable filter to remove residual cell debris form the released plant cell nuclear suspension. The nuclei are then concentrated in the annular flow centrifuge and the enriched suspension of the nuclei is then digested in tubes on a Peltier heater block to release the genomic DNA contained therein.



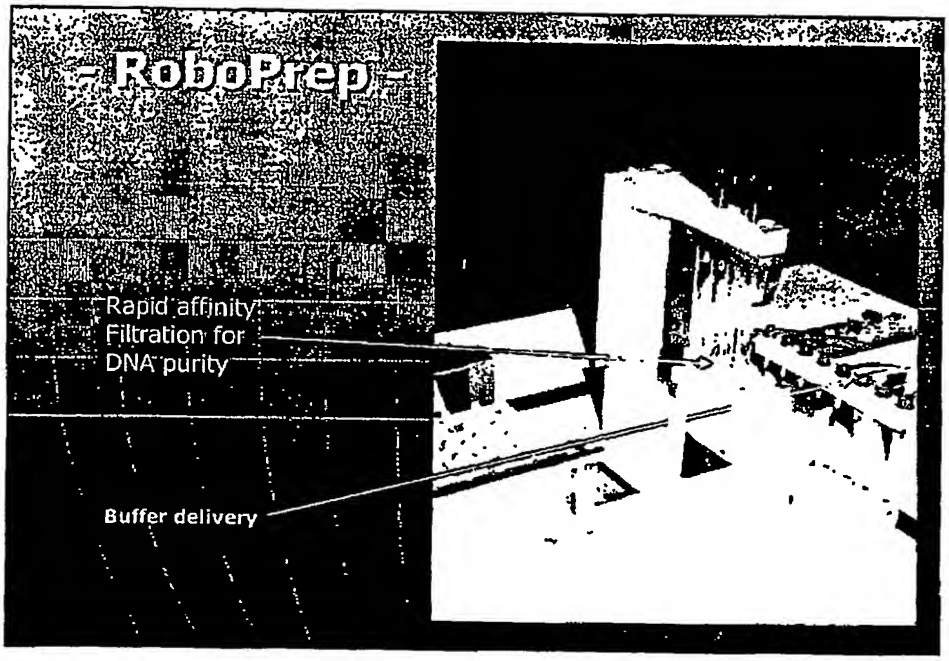
EXAMPLE 21

[0145] Elevated view of the flush flow annular centrifuge with collection arm and head. The arm and collection head permits the positive pumped collection of separated and partitioned fractions from the top of the centrifugal column within the annulus of the centrifuge rotor, whilst the rotor continues to rotate.



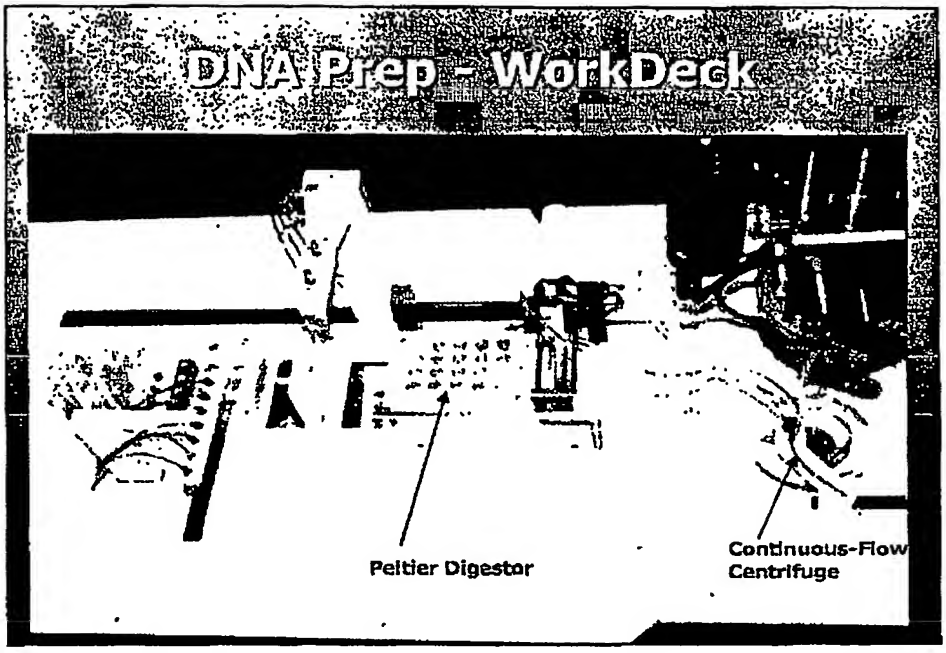
EXAMPLE 22

[0146] Horizontal view of the DNA binding resin columns and apparatus to load clarified plant nuclear digests, to add column washing solutions and to add DNA elution buffer



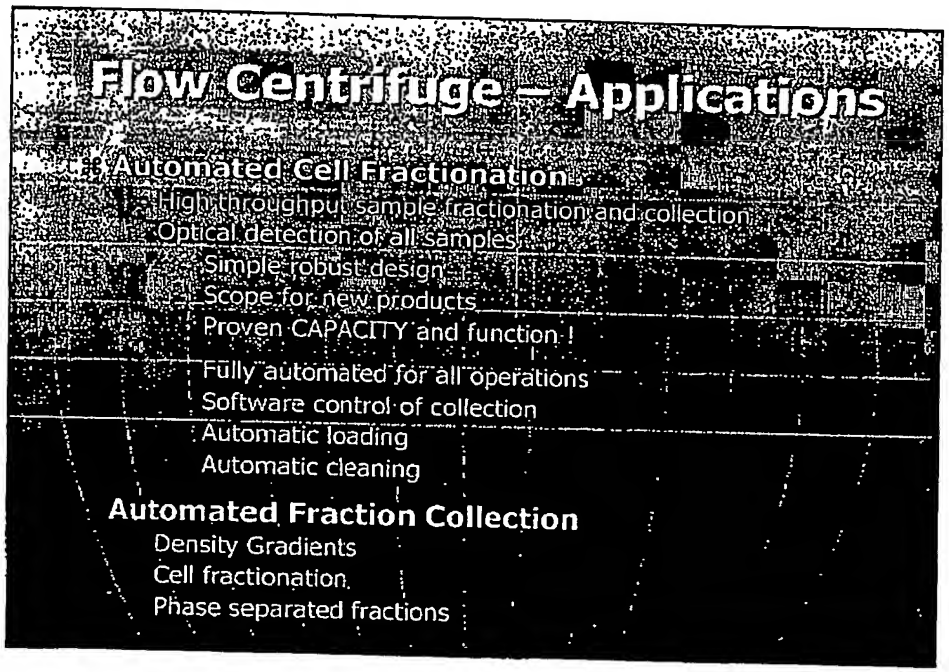
EXAMPLE 23

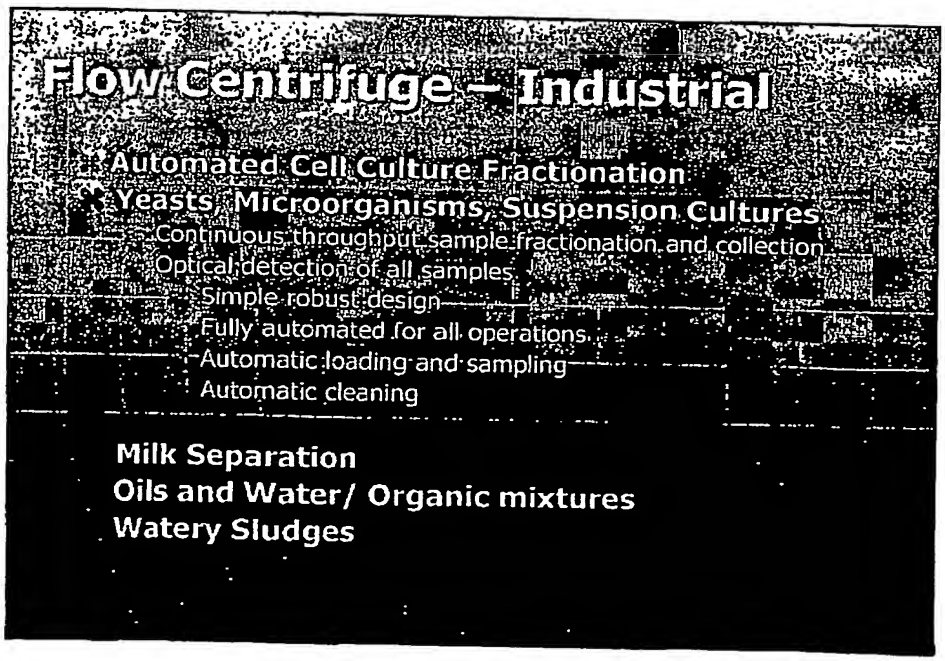
[0147] Overhead view of the connection between the flow centrifuge and the Peltier heater digestor. Enriched cell nuclei are pumped from the centrifuge rotor and mixed with a protease enzyme before being incubated in tubes on the Peltier block. The action of the protease inactivates residual DNA nuclease activities and releases the genomic DNA from the nuclei matrix and from DNA binding proteins.



EXAMPLE 24

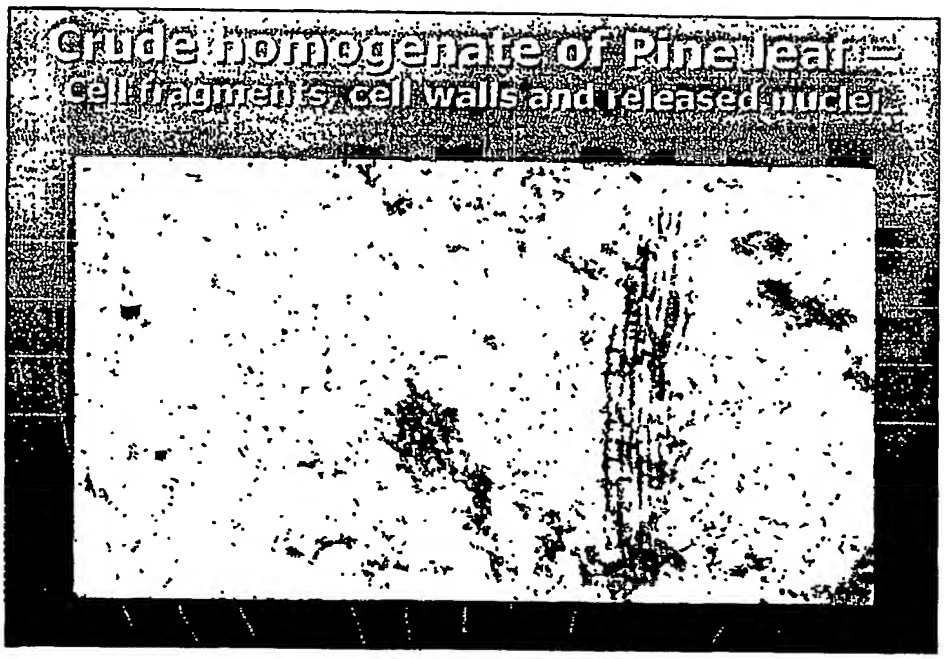
[0148] The Flow Centrifuge can be used to separate cells and cell organelles, density gradients and phase solution systems, sludges and suspensions. The positive collection arm and head ensure that complete fractions are collected with minimal cross contamination.





EXAMPLE 25

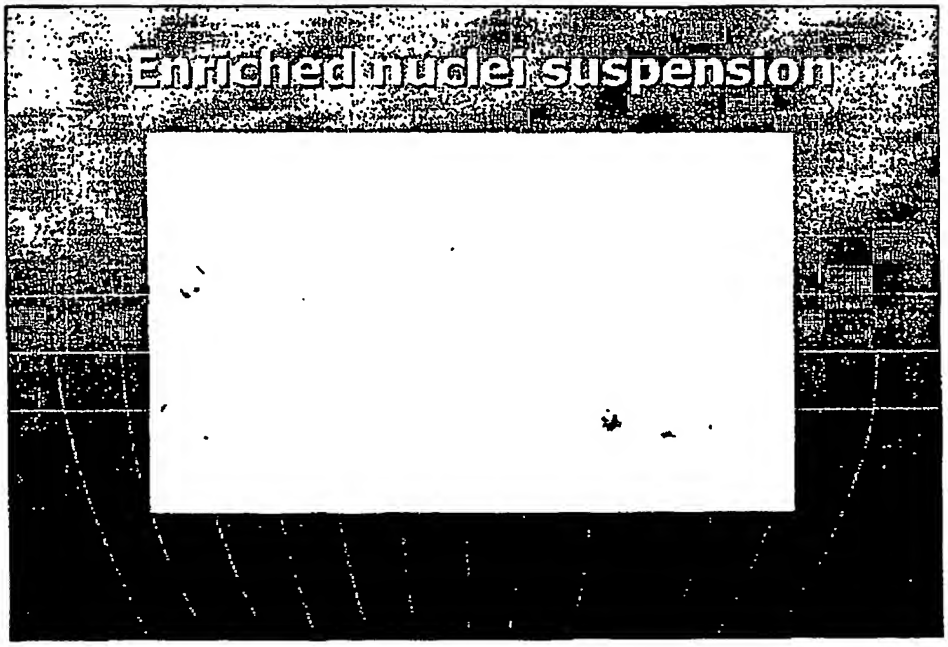
[0149]WORKING PREPARATIONS: The crude leaf homogenate that emerges fro m the tissue homogenizer contains whole leaf masses, cell fragments, cell particulate matter and plant cell walls as well as liberated cell nuclei



Microscopic view of the crude cell homogenate. The view is entirely filled with macerated cell walls. Free cell nuclei are not identifiable.

EXAMPLE 26

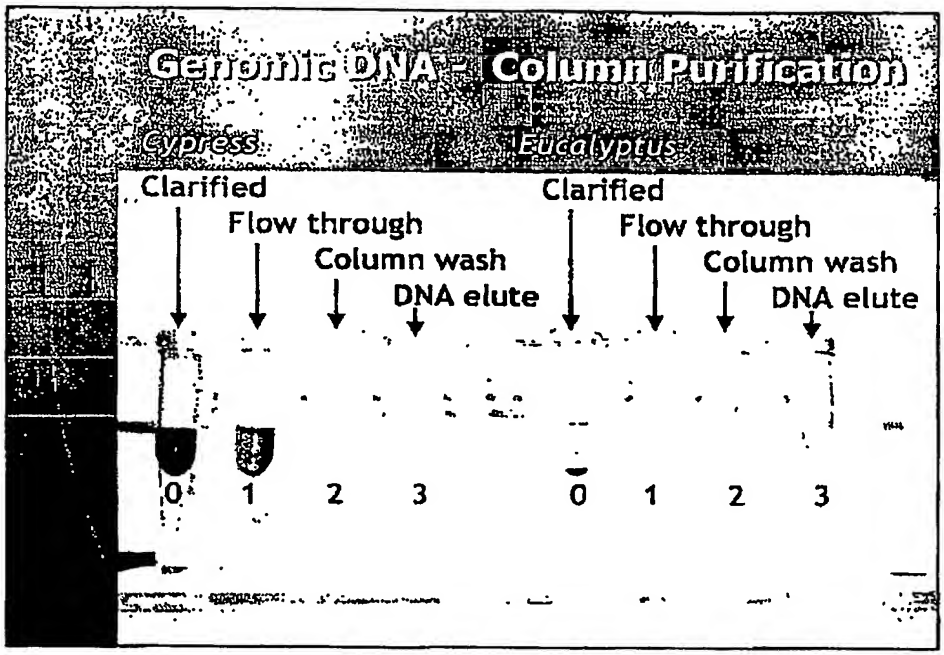
[0150]WORKING PREPARATIONS: The plant cell nuclei suspension recovered from the annular centrifuge following phase separation is enriched in cell nuclei compared to residual cell debris and cell wall fragments. Large cell masses are also essentially absent. The phase separation also removes the majority of the plant cell soluble materials such as phenolic compounds and storage polysaccharides and proteins, away from the free nuclear suspension.



Microscopic view of the enriched nuclear suspension. The large spheres are predominantly free cell nuclei.

EXAMPLE 27

[0151] Solutions eluted from the DNA binding resin columns



Solution eluted off from the DNA binding resin columns.

0, crude clarified [centrifuged] digest of genomic DNA from plant nuclei;

1, flow through solution of clarified digest recovered off resin column;

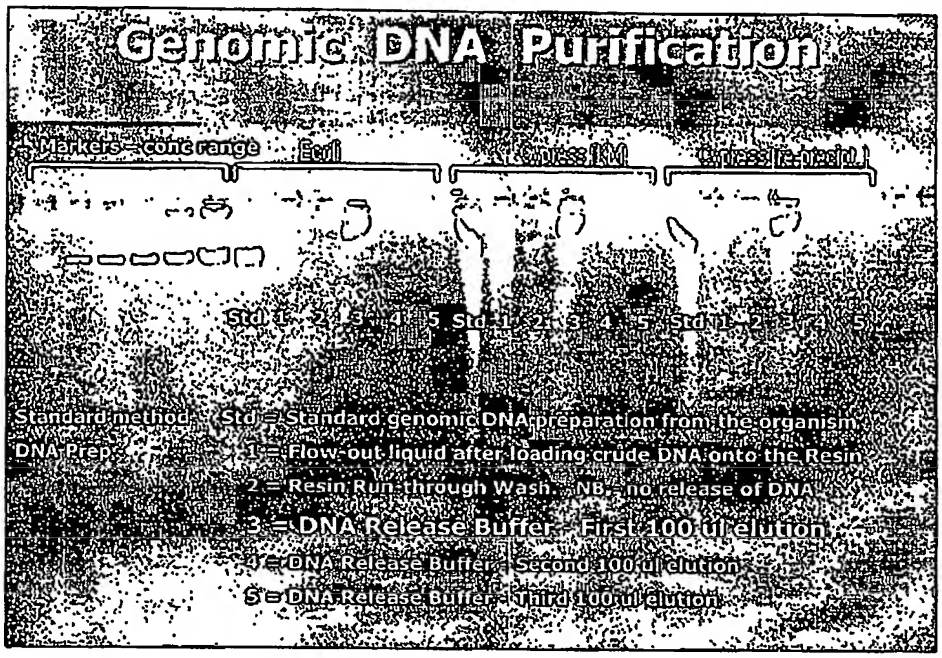
2, flow through of column washing buffer following step 1;

3, flow through of DNA elution solution to recover genomic DNA from the column.

Note that any residual coloured phenolic solution in the crude clarified digest solution does not attach to the DNA binding resin, but is washed away with the flow through solutions 1 and column wash solutions 2. The eluted genomic DNA in solution 3 is free of all residual phenolic contaminants.

EXAMPLE 28

[0152]Analysis of solutions eluted from the DNA binding resin columns.



The solution eluted from the DNA binding resin were assayed directly for DNA content by loading equal volume aliquots of each column fraction onto a DNA resolving agarose electrophoresis gel.

Std, standard phenol-chloroform purified genomic DNA from the organism.

1, flow through crude clarified following resin column loading. Most of the genomic DNA binds to the resin column.

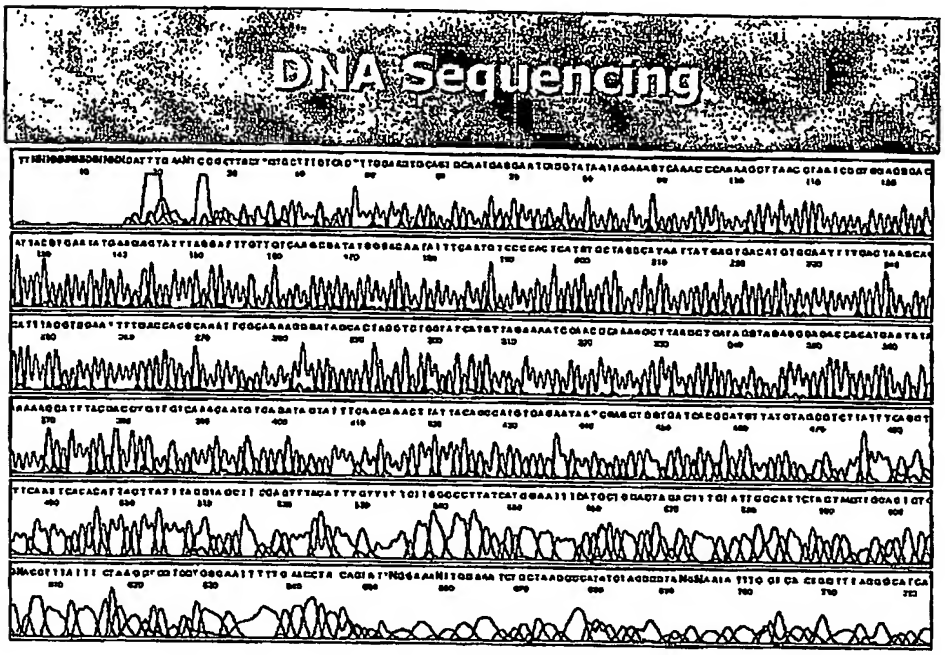
2, the resin column wash through buffer eluate is free of any released DNA.

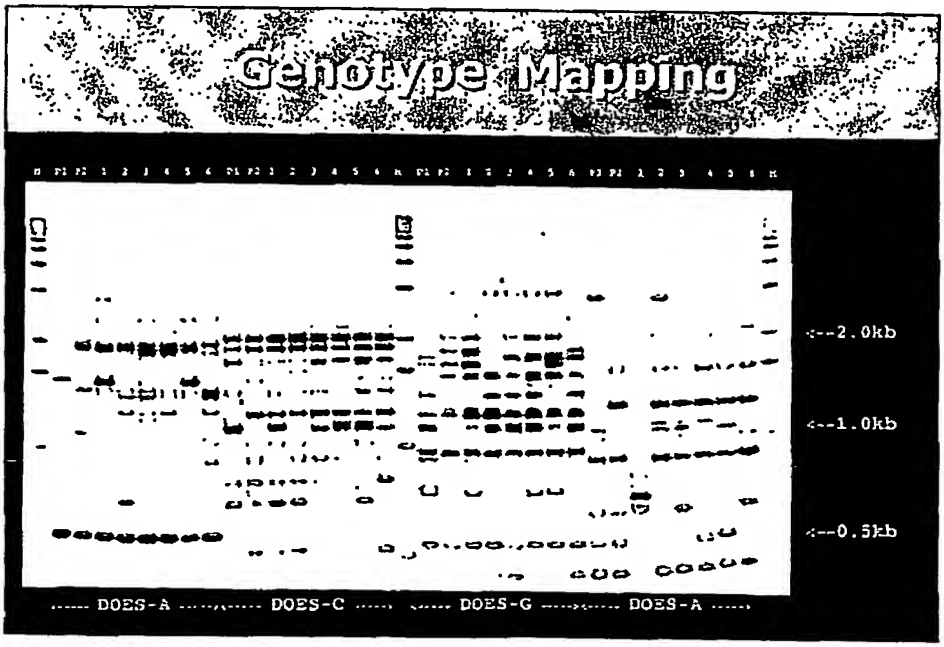
3, the DNA is released from the binding resin.

4 and 5, second and third elution releases of DNA from resin column. Note that little DNA is released with either the second or third elution steps as all DNA has been released in 3.

EXAMPLE 29

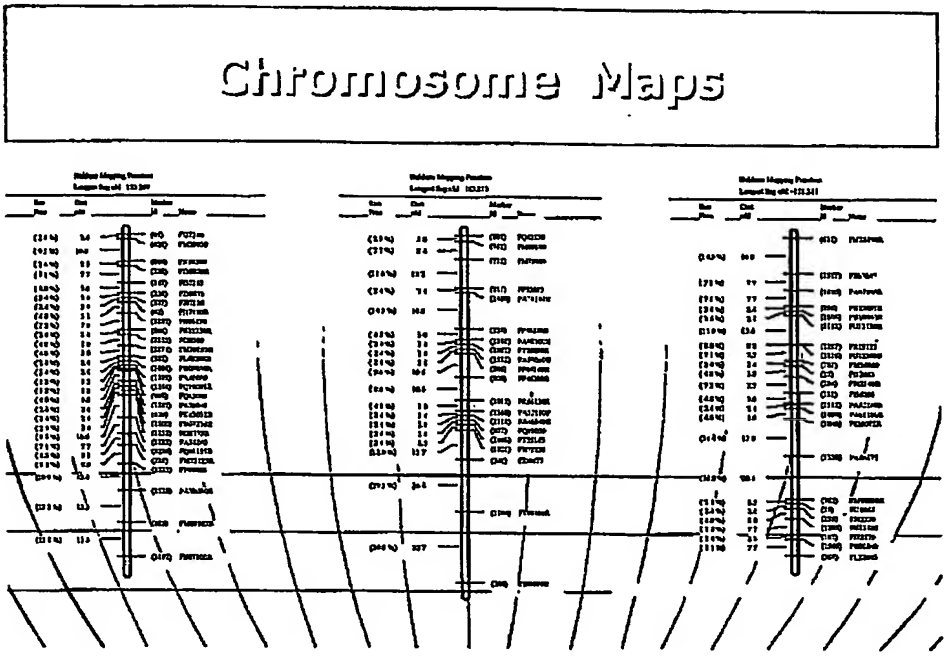
[0153]Analysis of genomic DNA eluted from the DNA binding resin columns. The genomic DNA is of high quality and can be employed using standard molecular biological procedures for DNA sequence analysis and for molecular marker genotype mapping.

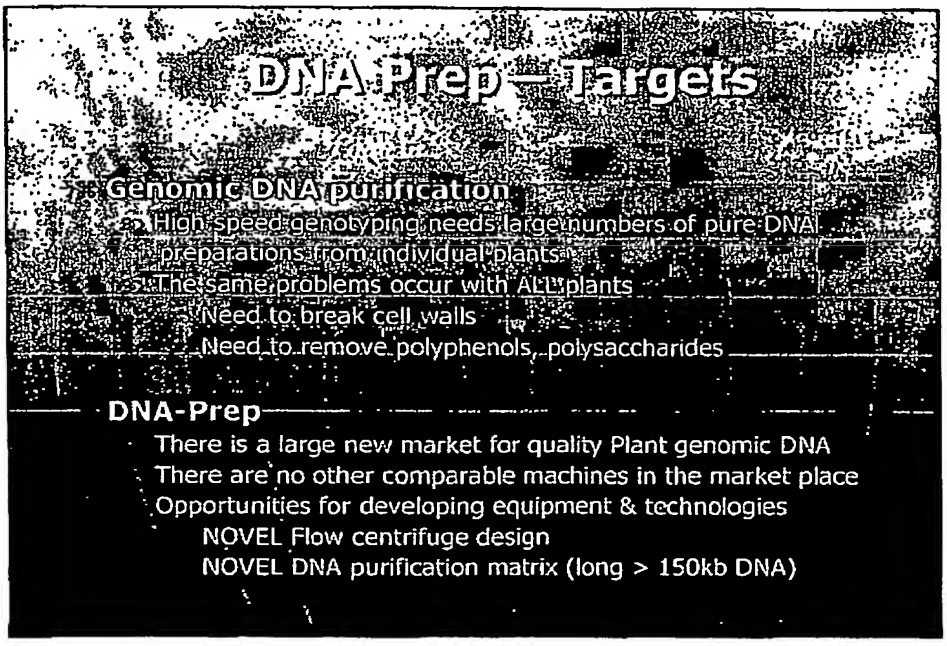




EXAMPLE 30

[0154] Utilization of genomic DNA recovered from the DNA preparation apparatus and procedure. The genomic DNA is of high quality and can be employed for molecular marker genotype mapping to construct chromosomal maps of linked markers. This invention solves problems for DNA purification which are common to all plants.





CLAIM

[0155]What is claimed is:

CENTRIFUGE

[0156]1. A method of serially *centrifuging* a plurality of samples, the method comprising the steps of:

introducing a sample into at least one container, wherein the container comprises at least one opening and a chamber;

rotating of the container around a single longitudinal axis of the chamber of the container, said rotating subjecting the sample in the container to centrifugal force sufficient to separate the sample into at least two components;

wherein said container comprises a rotor shell defining an inner separation chamber cavity, said rotor shell defining an orifice to discharge sample to said rotor shell and to collect sample from said rotor shell.

[0157]2. A method of serially centrifuging a plurality of samples, the method comprising the steps of:

a tubular arm or tubular arms having a plurality of passages and being constructed and arranged to supply the sample and to collect sample, said tubular arm or arms orientated perpendicular to the axis of rotation and centred at the axis of rotation of said inner cavity at the centre of said rotor opening;

said rotating separation chamber and a tubular arms perpendicular to the axis of rotation, said rotating causing rotation of the separation chamber without rotation of the tubular arms; said tubular arms are employed for the introduction of sample into the chamber, for the

addition of other components suitable for centrifugation into the chamber and for the withdrawal of sample and other components from the chamber;

said tubular arms are employed for the introduction of sample, or other components suitable for centrifugation, or for the withdrawal of sample and other components from the chamber whilst the chamber is rotating.

[0158]3. A method of serially centrifuging a plurality of samples, the method comprising the steps of:

a rotor shell defining an inner cavity, said rotor shell defining an inwardly extending baffle constructed and arranged to agitate and mix liquids contained within the rotor cavity.

4. The *method* of claim 1 or 2, wherein said tubular arms constructed and arranged to deliver the sample to and sample from said inner cavity through both a *supply flow* path and a *collection flow* path and a *waste flow* path with the said tubular arms;

wherein said *supply flow* path includes said rotor orifice and said inner separation cavity;

wherein said *collection flow* path includes said rotor orifice and said inner separation cavity; and

wherein said *waste flow* path includes said rotor orifice and said inner separation cavity;

wherein said inner cavity is constructed and arranged to discharge the sample received from both said *supply flow* path and said *collection flow* path through said tubular arm;

wherein said inner cavity is constructed and arranged to discharge the fluid and fluidic suspensions received from *waste flow* path out said tubular arm.

[0159]5. The *centrifuge* of claim 4, wherein:

said tubular arm has a supply passage and supply ports and a collection passage and collection ports defined therein;

said supply port is in fluid communication with a plurality of fluid supplies connected by said

supply *flow* path;

said supply ports and collection ports occur at the end of the tubular arms distal to the axis of rotation;

said collection passage is connected to said collection ports;

said collection port is in fluid communication with a plurality of fluid collection chambers connected by said collection *flow* path;

said tube arm has a *waste wash* supply passage and *wash supply* ports and a *waste collection* passage and *waste collection* ports defined therein;

said *wash supply* passage is connected to said *wash separation* ports;

said *waste collection* port is in fluid communication with a plurality of *waste collection* fluid supplies connected by said *waste collection flow* path;

said *wash supply* ports and *waste collection* ports occur at the end of the tubular arms distal to the axis of rotation;

[0160]6. The method of claims 1 or 2, wherein the container comprises at least one opening for introduction of a sample and other suitable components and for the withdrawal of a separated sample component whilst the container is rotating;

[0161]7. The method of claim 3 or 6, wherein the container is rotated prior to introducing the sample;

[0162]8. The method of claim 3 or 6, wherein the container is rotated prior to separating the sample;

[0163]9. The method of claim 3 or 6, wherein the container is rotated after separating the sample;

[0164]10. The method of claims 6 or 7 or 8, wherein other components suitable for separation of the sample by centrifugation are introduced into the container prior to the introduction of the sample;

[0165]11. The method of claims 6 or 7 or 8, wherein other components suitable for separation of the sample by centrifugation are introduced into the container during the introduction of the sample;

[0166]12. The method of claims 2 or 4 or 6, wherein the container is rotated during the withdrawal of the separated component of the sample;

[0167]13. The method of claim 6, wherein the method comprises collecting a component of the sample that flows through the opening of the container through the tubular passage;

[0168]14. The method of claim 2 or 3 or 6, wherein the container is rotated prior to cleaning the container of the previous sample;

[0169]15. The method of claims 6 or 14, wherein other components suitable for cleaning of the chamber are introduced into the container prior to the introduction of the sample;

[0170]16. The *method* of claim 1, wherein said container is constructed from a translucent or signal transmitting material such that defined fluid regions and particulate materials, menisci and other interfaces may be detected optically, spectrally, electrically, rheostatically, or by other physical means that is appropriate to detect the contents of the rotating chamber;

[0171]17. The *method* of claim 1 or 2 or 3, wherein said collection arms are constructed from a translucent or signal transmitting material such that defined fluid regions and particulate

materials, menisci and other interfaces may be detected optically, spectrally, electrically, rheostatically, or by other physical means as is appropriate to detect the contents of the collection flow paths;

[0172]18. The *method* of claim 1 or 2 or 3, wherein said supply arms are constructed from a translucent or signal transmitting material such that defined fluid regions and particulate materials, menisci and other interfaces may be detected optically, spectrally, electrically, rheostatically, or by other physical means as is appropriate to detect the contents of the supply flow paths;

[0173]19. The *method* of claim 1 or 2 or 3, wherein said waste arms are constructed from a translucent or signal transmitting material such that defined fluid regions and particulate materials, menisci and other interfaces may be detected optically, spectrally, electrically, rheostatically, or by other physical means as is appropriate to detect the contents of the waste flow paths;

[0174]20. The detectors of claim 16 or 17 or 18 or 19 may be connected to logical processor and control apparatus that direct the motions of the collection and supply and waste tubular arms;

[0175]21. The logical processor and control apparatus of claim 20 may direct the motions and activities of fluid transport mechanisms that regulate the directional motion of fluid materials within said fluid collection arms, said fluid supply arms and said fluid waste arms;

[0176]22. A logical processor and control apparatus of claim 20 may be used to control the speed (angular velocity) and the direction of rotation of the rotating container;

[0177]23. The *method* of claim 2 or 3, wherein said tubular arm has one or more openings defined therein through which the fluid from said *collection flow* path flows

[0178]24. The *method* of claim 2 or 3, wherein said tubular arms each possess a stator attached to motion apparatus capable of controlled linear motion or the tubular arm;

[0179]25. The method of claim 20 or 23 or 24, wherein said linear motion of said tubular arms may be regulated by a logical processor and control apparatus;

[0180]26. The *method* of claim 2 or 3, wherein said *supply flow* path and a *collection flow* path and a *waste flow* path in proportion to *flow* rates in said *supply flow* path and said *collection flow* path and said *waste flow* path;

[0181]26. The method of claim 20 or 23 or 26, wherein said flow rates may be regulated by a logical processor and control apparatus.;

[0182]27. The *centrifuge* of claim 1, wherein said rotor shell includes an upper rotor shell portion and a lower rotor shell portion mated with said upper rotor shell portion; wherein said *centrifuge* of claim 1, further comprising an annular assembly provided in said separation cavity around said tubular arms; wherein
said *centrifuge* of claim 1, further comprising a *supply* tubular arm extending said fluid passage within said tube, said tube defining a *supply* passage constructed and arranged to deliver the fluid to said fluid passage.

[0183]28. The *centrifuge* of claim 27, wherein said tubular arm has one or more supply ports defined therein to supply the fluid from said *supply* passage to said fluid passage.

[0184]29. The *centrifuge* of claim 1, further comprising a *collection* tubular arm extending said fluid passage within said tube, said tube defining a sample *collection* passage constructed and arranged to collect the fluid from said fluid passage; whereas
said *centrifuge* of claim 1, wherein said tubular arm has one or more supply ports defined therein to collect the fluid from said sample *collection* passage; whereas
said *centrifuge* of claim 1, further comprising a waste tubular arm extending said fluid passage within said tube, said tube defining a supply passage constructed and arranged to supply the wash fluid from said fluid passage; whereas
said *centrifuge* of claim 1, further comprising a waste tubular arm extending said fluid passage within said tube, said tube defining a collection passage constructed and arranged to collect the waste fluid and particulates from said fluid passage;

[0185]30. The *centrifuge* of claim 29, wherein said waste tubular arm has one or more supply ports defined therein to collect the fluid and particulates from said fluid passage.

[0186]31. The *centrifuge* of claim 2, wherein:
said tubular arms extend along a longitudinal axis; and
said longitudinal axes are oriented perpendicular to said rotational axis of said centrifuge bowl.

[0187]32. The *centrifuge* of claim 10, wherein said supply tubular arms can supply the fluid to said centrifuge bowl along a *flow* path adjacent to the inner wall of the annular rotor bowl; whereas of said *centrifuge*, wherein said *supply* or *waste* tubular arms can aid in the mixing of the fluid and particulate contents of the rotor bowl during rotational motion of said bowl.

[0188]33. The *centrifuge* of claim 2, wherein:
said tubular arms extend along a longitudinal axis; and

said longitudinal axes are oriented perpendicular to said rotational axis of said centrifuge bowl.

[0189]34. The *centrifuge* of claim 10, wherein said *supply* tubular arms can supply the fluid and particulate contents to said centrifuge bowl along a *flow* path adjacent to the inner radial wall of the annular rotor bowl;

wherein, said *collection* tubular arms can collect the fluid and particulate contents from said centrifuge bowl along a *flow* path adjacent to the inner radial wall of the annular rotor bowl;

wherein, said waste tubular arms can collect the fluid and particulate contents from said centrifuge bowl along a *flow* path adjacent to the inner radial wall of the annular rotor bowl;

[0190]35. The *centrifuge* of claim 2, wherein:

said tubular arms extend along a longitudinal axis; and;

said longitudinal axes are oriented perpendicular to said rotational axis of said centrifuge bowl; wherein

said tubular arms extend along a longitudinal axis, and;

said longitudinal axes are oriented perpendicular to said rotational axis of said centrifuge bowl;

[0191]36. The *centrifuge* of claim 3, wherein said *supply* tubular arm is constructed and arranged to mix and discharge together the fluid from said *supply* fluid *flow* paths and said sample input *flow* path out said supply ports;

said *supply* tubular arm defining a single fluid supply passage to supply fluid to the *centrifuge,* said tubular arm defining one or more supply ports that are in fluid communication with said fluid supply passage, said tubular arm defining one or more bypass ports that are in fluid communication with said fluid supply passage;

[0192]37. A *centrifuge* of claim 1 comprising, a rotor shell defining an inner cavity in which a baffle is positioned annularly between the inner radial perimeter and the rotor axis;
said baffle comprising vanes perpendicular to the centripetal vector of the rotor, wherein said baffle is constructed and arranged to mix fluid within said separation portion of the rotor shell;

[0193]38. The method of claims 1, 2, 3 or 4, wherein the tubular arm or arms are adapted for removable insertion in one orifice of the rotor and horizontal motion perpendicular to both the axis of rotation of the rotor and to the centripetal vector of the rotor;

[0194]39. A method of sequentially centrifuging a plurality of samples, the method comprising the steps of:
The method of claim 14, introducing a first sample into the rotating chamber, wherein said introducing of the sample is accomplished by transfer of a sample from a well of a sample substrate source comprising a plurality of source wells, and;
said source well, that may be 1 well; preferably may be 2 wells; more preferably may be 3 wells; more preferably may be 4 wells; more preferably may be 5 wells; more preferably may be 6 wells; more preferably may be 7 wells; more preferably may be 8 wells; more preferably may be 9 wells; more preferably may be 10 wells into said chamber, and;
wherein, the method of claim 7, rotating the chamber around a single longitudinal axis of the chamber, said rotating of the chamber subjecting the sample in the chamber to centrifugal force sufficient to separate the sample into plurality of components comprising at least two components; and;

The method of claims 5 and 6, collecting a separated sample component or components from said rotor chamber, and;

The method of claims 5 and 6, washing residual sample component or components from said rotor chamber, and;

The method of claims 5 and 6, washing residual sample components from said tubular supply arms, and;

The method of claims 5 and 6, washing residual sample components from said tubular collection arms, and;

The method of claims 5 and 6, washing residual sample components from said tubular waste collection arms, and;

The method of claim 32, the introduction of a second sample into the rotating chamber.

MILL

[0195]40. A method of serially *milling* a plurality of samples, the method comprising the steps of;

introducing a sample into at least one chamber, wherein the chamber comprises at least one opening and a rotor and a stator and a feeder;
wherein said rotor and stator comprise each a plurality of knife edges;
wherein rotating of the rotor around a single longitudinal axis of the chamber, said rotating subjecting the sample in the chamber to shear force sufficient to comminute the sample into at least two components; yet more preferred a multitude of cellular and subcellular components;
wherein the sample may flow along the knife edged conduit of the rotor and as the plurality

of knife edges of stator pass by the sample is able to move and rotate;

said chamber defining at least two orifices to discharge sample to said mill and to collect sample from said mill after comminution;

wherein said feeder may be helical

wherein said feeder is contiguous with the rotor;

wherein rotating of the feeder around a single longitudinal axis of the chamber, said rotating subjecting the sample in the chamber to longitudinal forces sufficient to migrate the sample towards the rotor to deliver the intact tissue sample to the rotor;

[0196]41. A method of serially milling a plurality of samples, the method comprising the steps of:

said *tissue mill* of claim 40, wherein said rotor and stator having complementary conical surfaces, each surface having one or the other of complementary shearing elements including a plurality of knife edges arranged on one said surface and substantially lying in a radial plane of said surface;

wherein said helical knife edged conduit disposed on the other of said surfaces;

wherein said stator may be located within the rotor;

wherein said rotor is preferred located within the stator;

wherein said stator is preferred include the plurality of knife edges, wherein said rotor has the helical knife edged conduit;

solution tubes having a plurality of passages and being constructed and arranged to supply sample buffer solutions and washing solutions, said tubes connecting to the milling chamber through the stator;

sample injector port being constructed and arranged to supply sample tissue, said port connecting to the milling chamber through the stator;

said port and tubes are employed for the introduction of sample into the chamber, for the

addition of other components suitable for milling into the chamber and for the withdrawal of sample and other components from the chamber;

said port and tubes are employed for the introduction of sample, or other components suitable for milling, or for the withdrawal of sample and other components from the chamber whilst the rotor and feeder are rotating in the milling chamber.

[0197]42. The method of claim 40 or 41, wherein the sample may flow along the knife edged conduit of the rotor and as the plurality of knife edges of stator pass by the tissue is able to move and rotate, being chopped into small pieces as the two knife edges pass;

[0198]43. The method of claim 40 or 41 or 42, wherein a suitable buffer may be fed into the tissue mill chamber to ensure to ensure flushing of the mill during operation;

wherein said buffer is preferred fed continuously during operation;

wherein said provision of buffer solution during milling may provide protection for the DNA that remains within the cell nucleus;

wherein said provision of buffer solution during milling may prevent tissue blockage;

[0199]44. The method of claim 40, wherein said feeder may be helical and act to deliver the intact tissue sample to the rotor;

wherein said helix may include knife-edges,

wherein said tissue fed into the rotating helix is chopped to a size to assist in the milling stage at the rotor and stator;

[0200]45. The method of claim 40, wherein the clearance between said rotor and stator may be varied across the length of the mill;

wherein, such that following delivery of tissue into the posterior [narrow end] of the mill there will be sufficient clearance for the partially intact plant tissues;

wherein, during operation of the tissue mill the forward movement of the tissue towards the anterior [wide end] of the mill and corresponding commination of the tissue occur across the mill working surfaces in which the clearance between the rotor and stator may diminish until a set minimum clearance between rotor and stator is achieved;

[0201]46. The method of claim 40, wherein said minimum clearance may be constant across the remaining portion of the mill working surfaces, providing for fine milling of the plant tissues prior to exit from the mill;

whereas, said volume available for occupation by the plant tissue within the any unit length chamber of the mill may be relatively constant across the length of the mill;

whereas, said volume may be provided by both the increasing surface area of the conical rotor and stator and the increasing width in the spacing between the helically aligned knife-edges on the rotor and radially aligned knife edges on the stator;

[0202]47. The method of claim 40, wherein the surfaces of said rotor and stator and feeder helix may be coated in a suitable metal surface treatment and coating;

wherein said surface treatment may include titanium nitride in particularly by the commercial Balinit process;

[0203]48. The method of claim 41, wherein said mill may be cleaned free of residual sample tissue following comminution by displacing of the feeder and rotor longitudinally along the longitudinal axis of the rotor so as to separate the conical rotor surfaces from conical surfaces of the stator; and

said surfaces of the milling chamber may be flushed free of residual sample by use of a suitable washing solution; and

wherein said rotor surface may be brought again into suitable juxtaposition with the stator by a reverse longitudinal displacement along the longitudinal axis of the rotor following cleaning;

[0204]49. The motors of claim 40 or 42 or 43 or 44 or 45 or 48 may be connected to logical processor and control apparatus that direct the motions of said components of said tissue mill for the supply of said tissue sample or for the supply of said buffer or for the rotation of said feeder and rotor or for the clearance between said rotor and stator or for the collection and supply of said sample following comminution or for the washing of said wastes from the surfaces of said tissue mill;

[0205]50. The logical processor and control apparatus of claim 49 may direct the motions and activities of fluid transport mechanisms that regulate the directional motion of fluid materials within said fluid collection tubes, said fluid supply tubes and said fluid waste tubes;

[0206]51. A logical processor and control apparatus of claim 49 may be used to control the speed (angular velocity) and the direction of rotation of the rotating rotor;

[0207]52. A method of manipulating nuclear material substantially as hereinbefore defined with reference to the specific embodiments described herein;

DATED THIS TWENTY-NINTH DAY OF SEPTEMBER 2003.

KEITH RICHARD MITCHELSON

by

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72 HIGHLAND TERRACE, ST LUCIA, QLD 4067

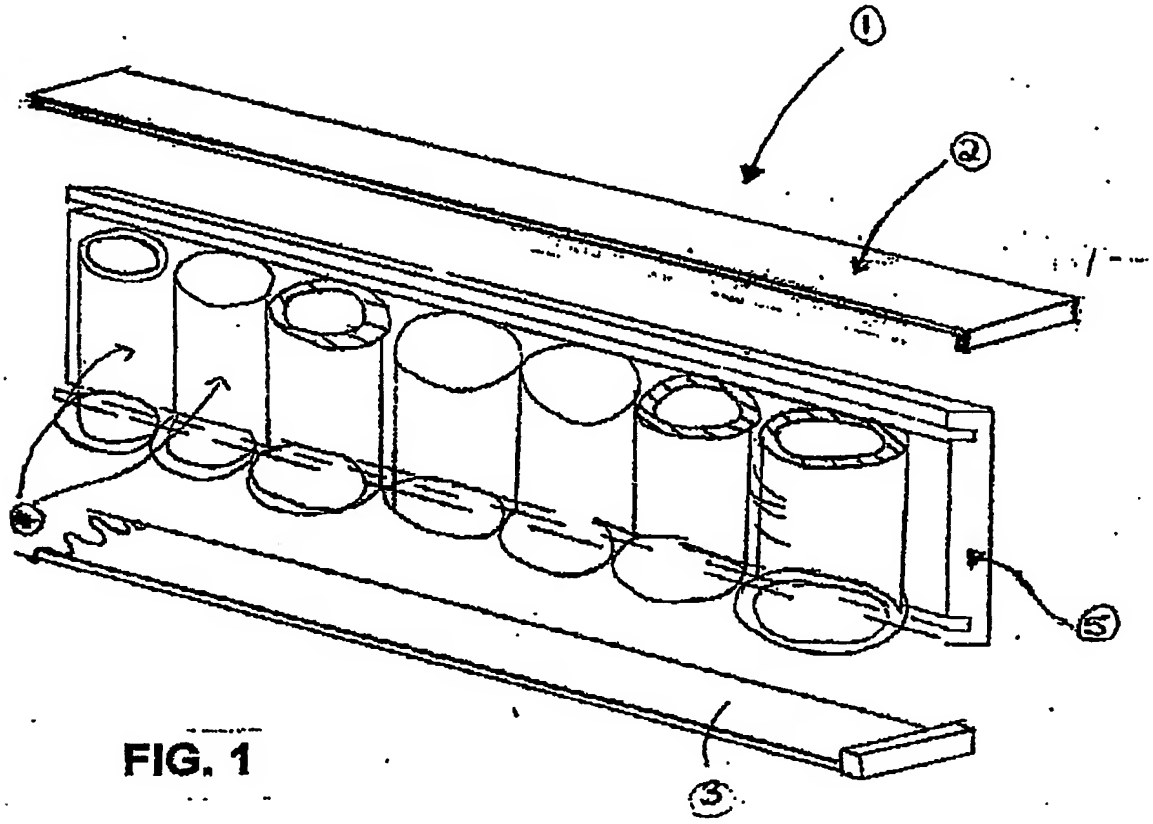


FIG. 1

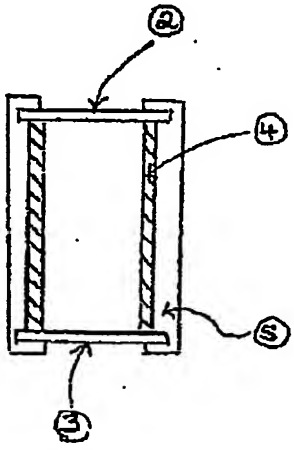


FIG. 1A

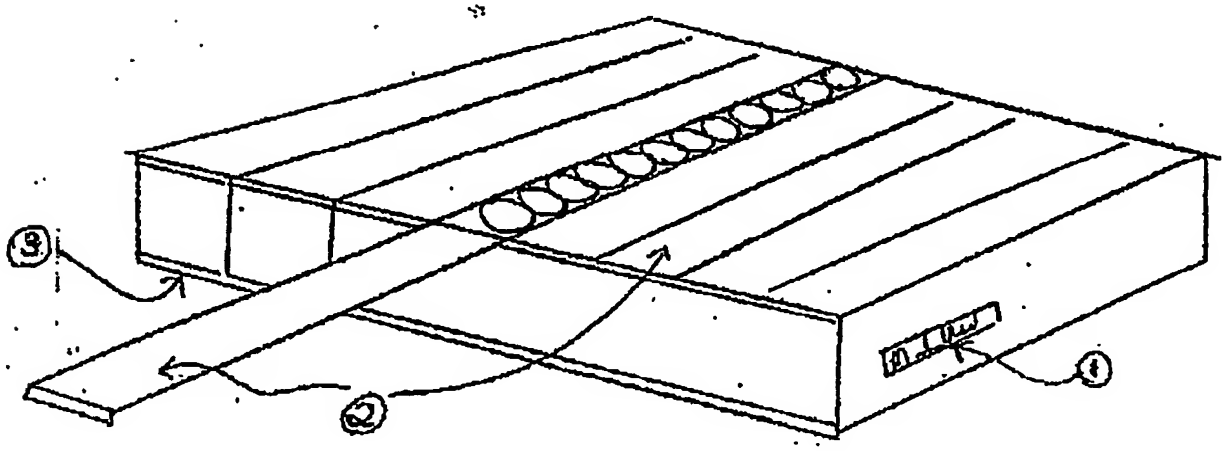


FIG. 2

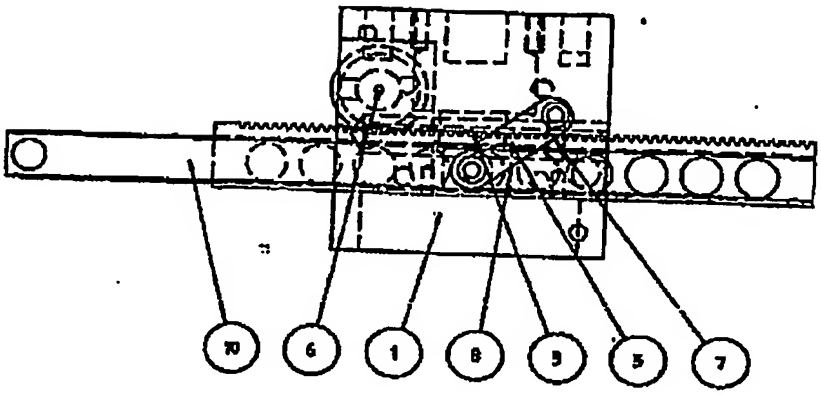


FIG. 3

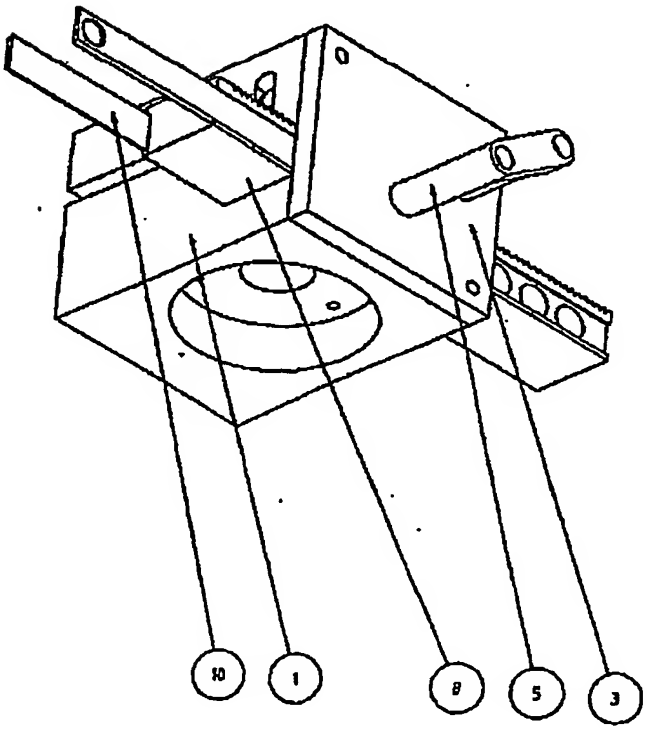


FIG. 3A

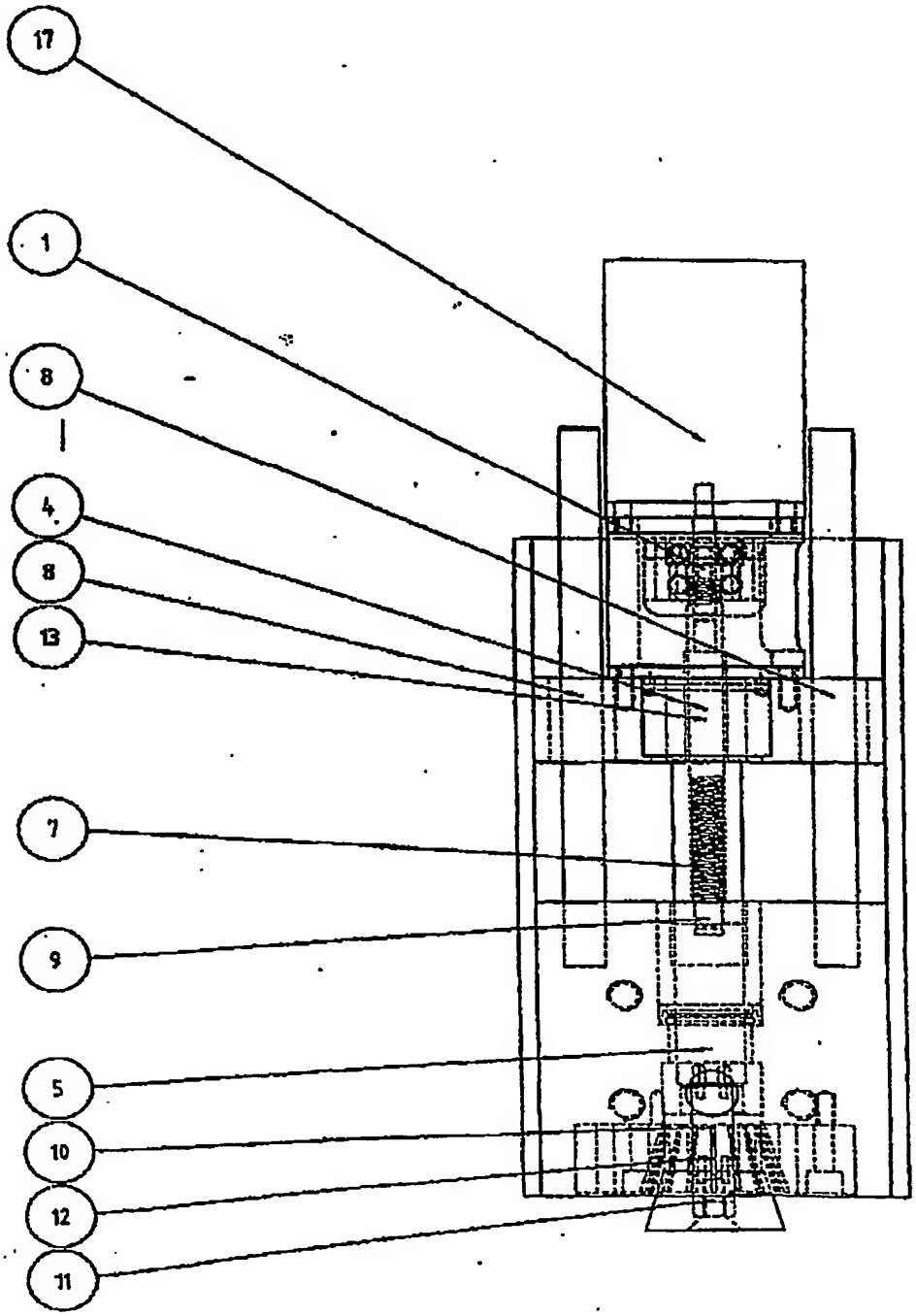


FIG. 4

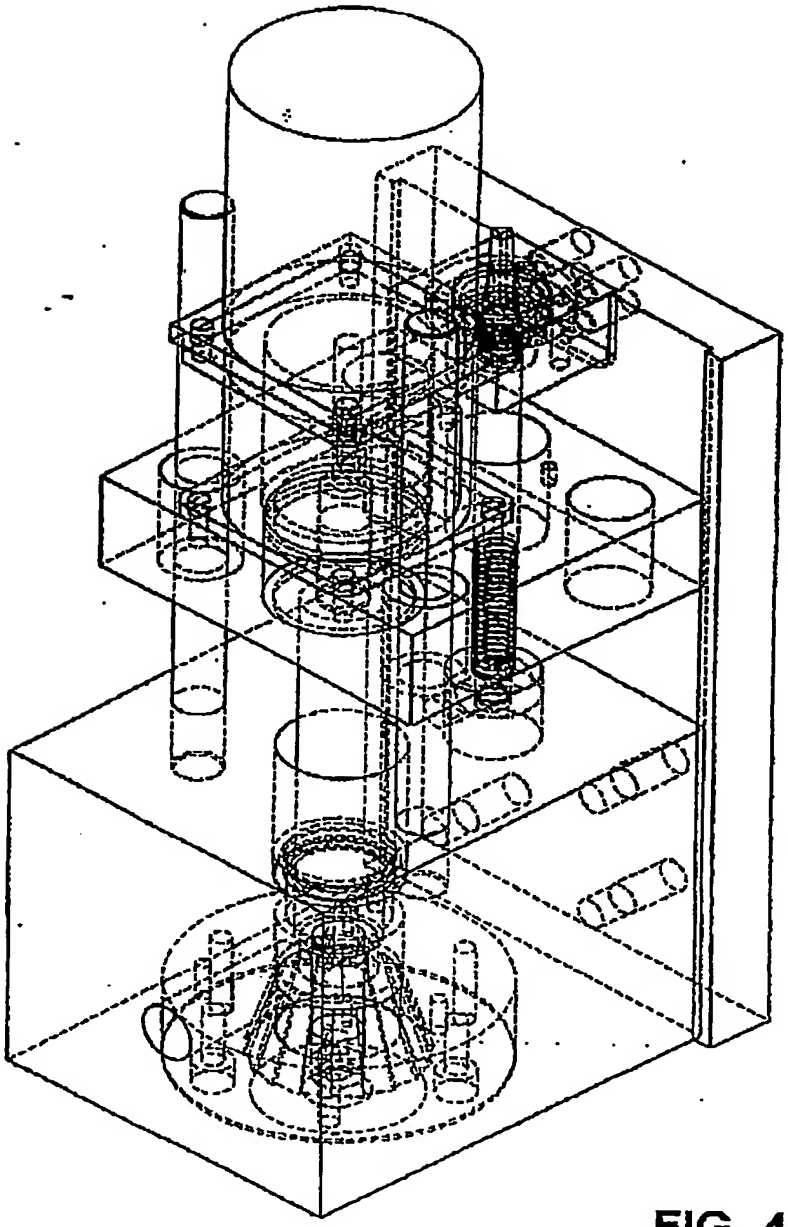


FIG. 4A

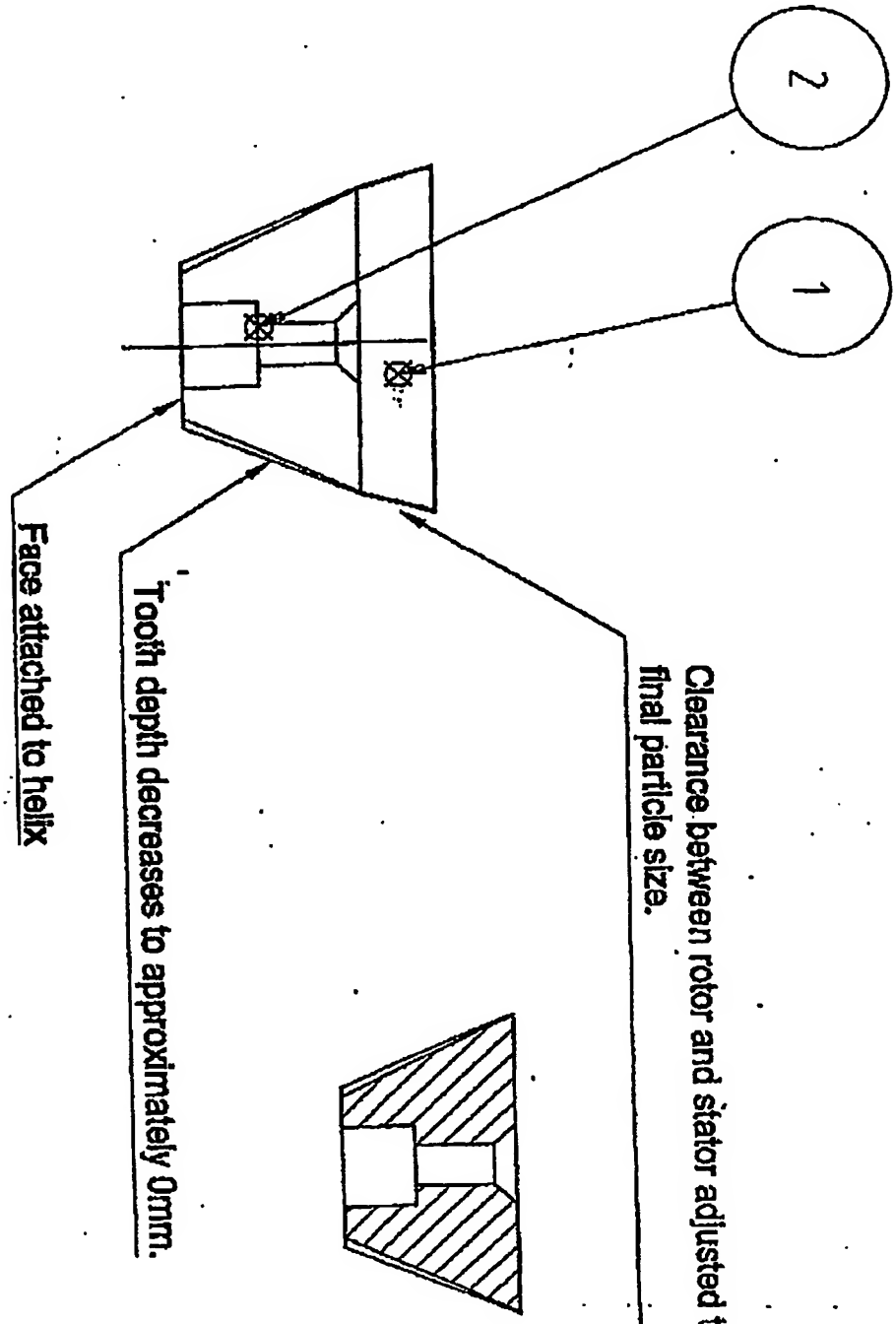
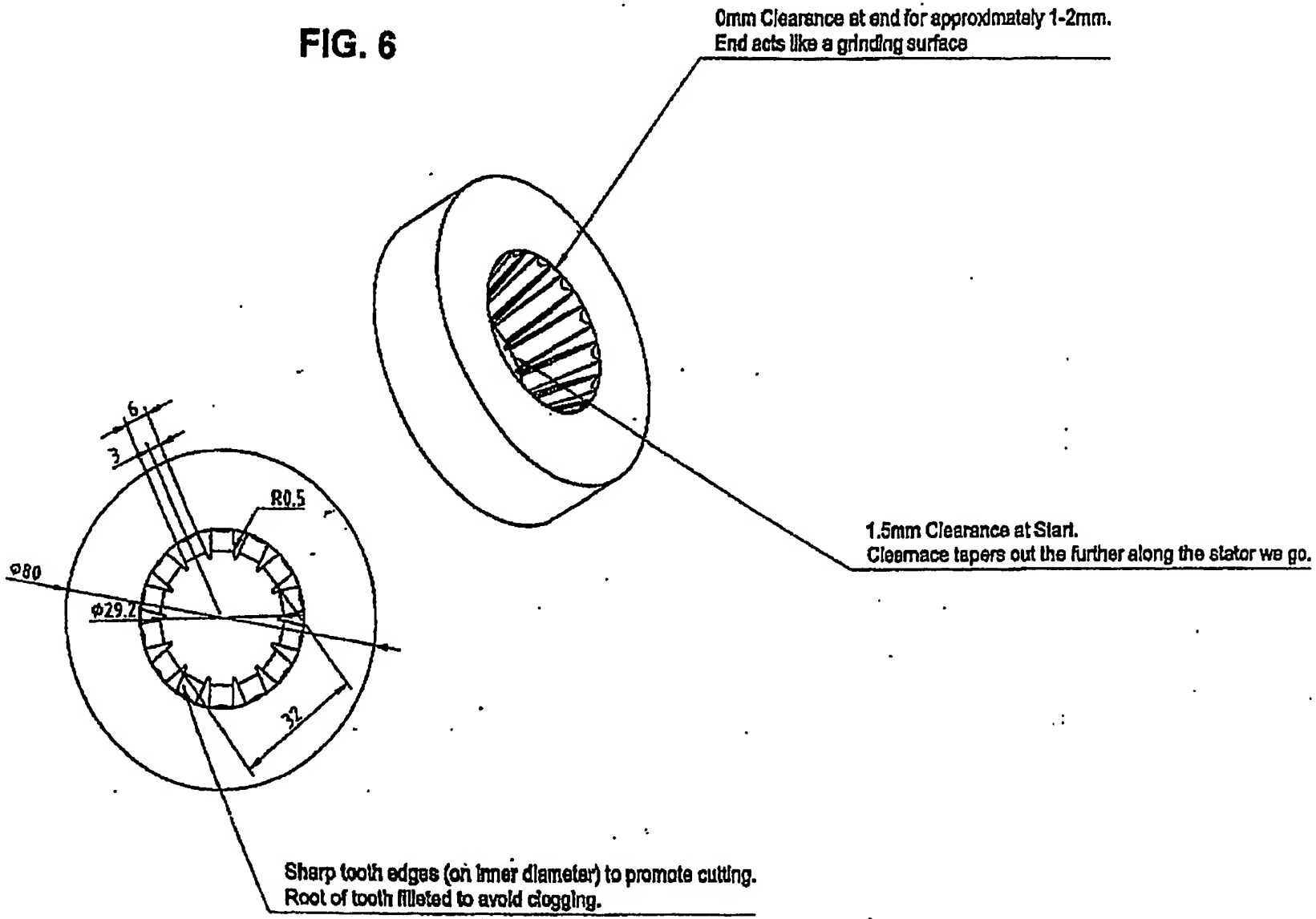

2
1
Clearance between rotor and stator adjusted to define final particle size.
Tooth depth decreases to approximately 0mm.
Face attached to helix

FIG. 5

FIG. 6


0mm Clearance at end for approximately 1-2mm.
End acts like a grinding surface
1.5mm Clearance at Start.
Clearnace tapers out the further along the stator we go.
6
3
R0.5
Φ80
Φ29.2
32
Sharp tooth edges (on inner diameter) to promote cutting.
Root of tooth filleted to avoid clogging.

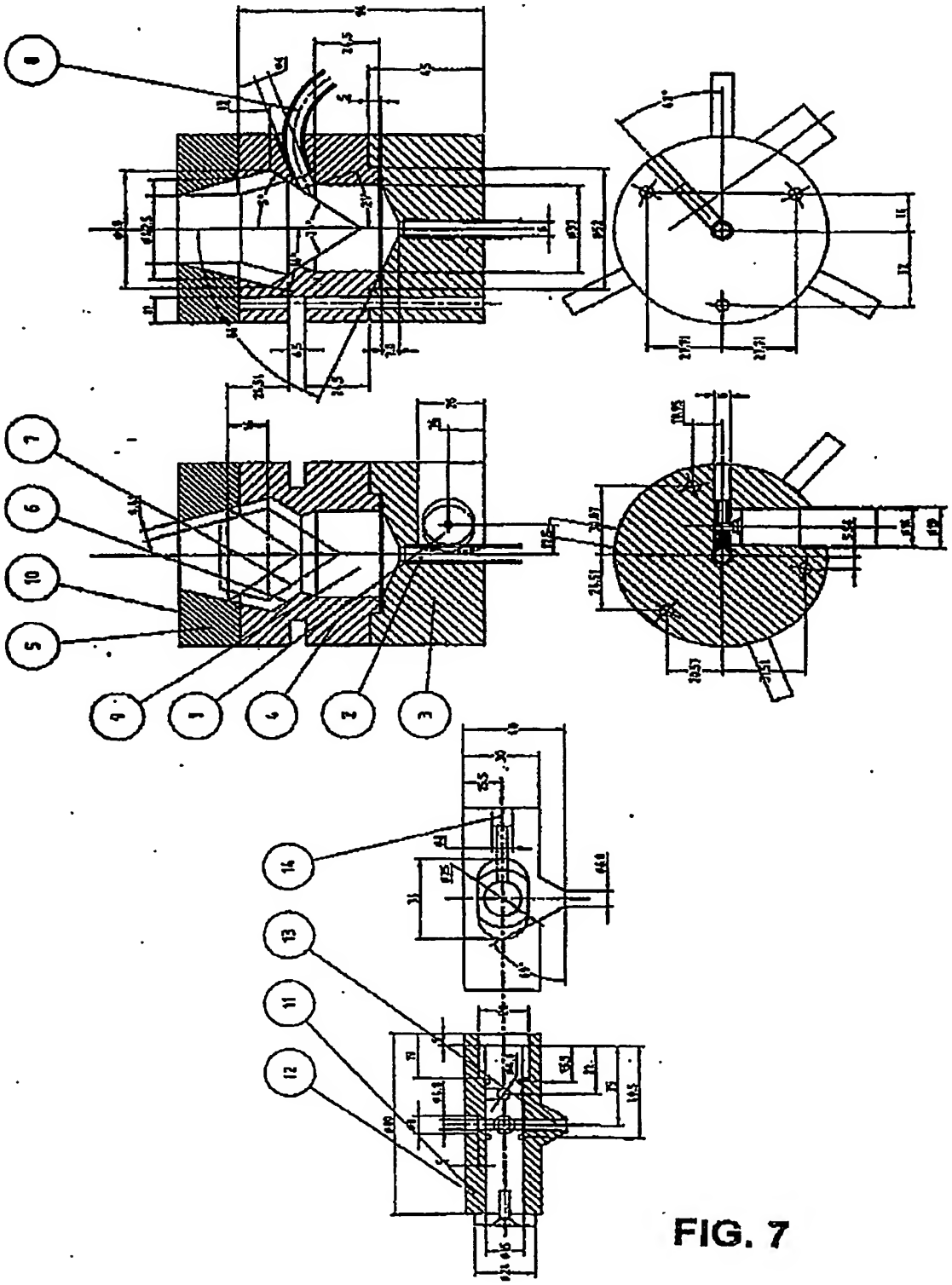


FIG. 7

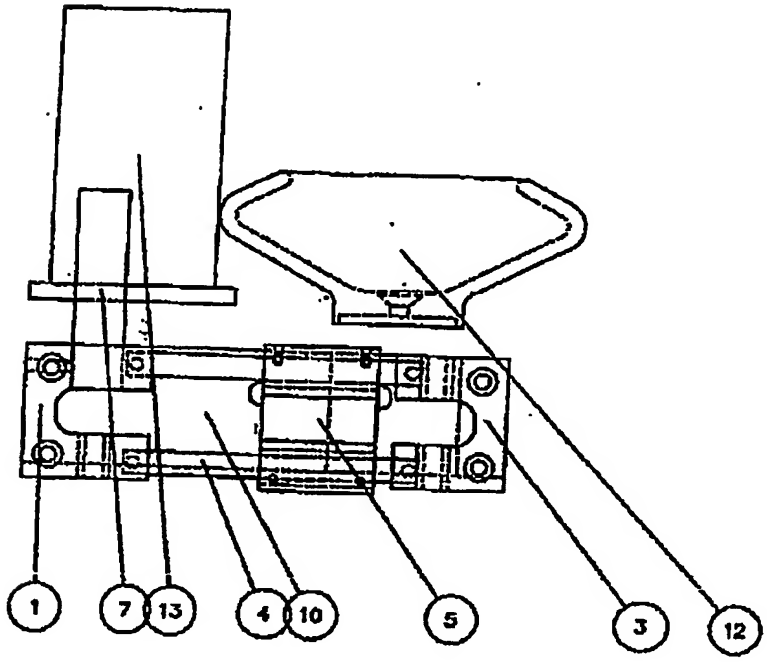


FIG. 8

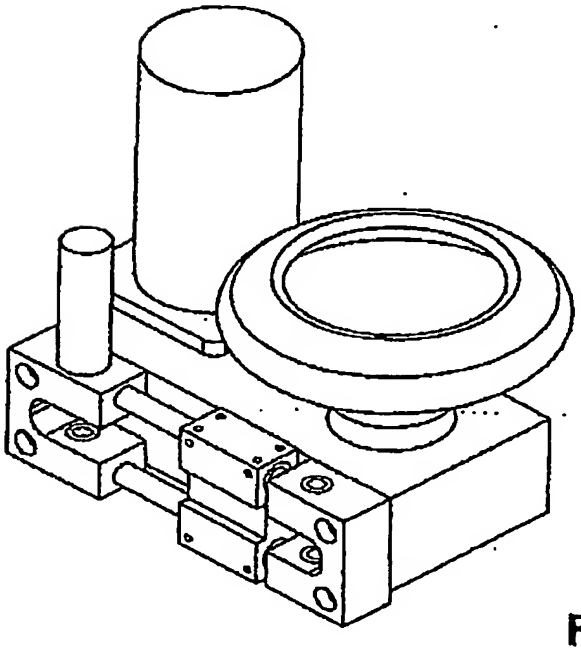


FIG. 8A

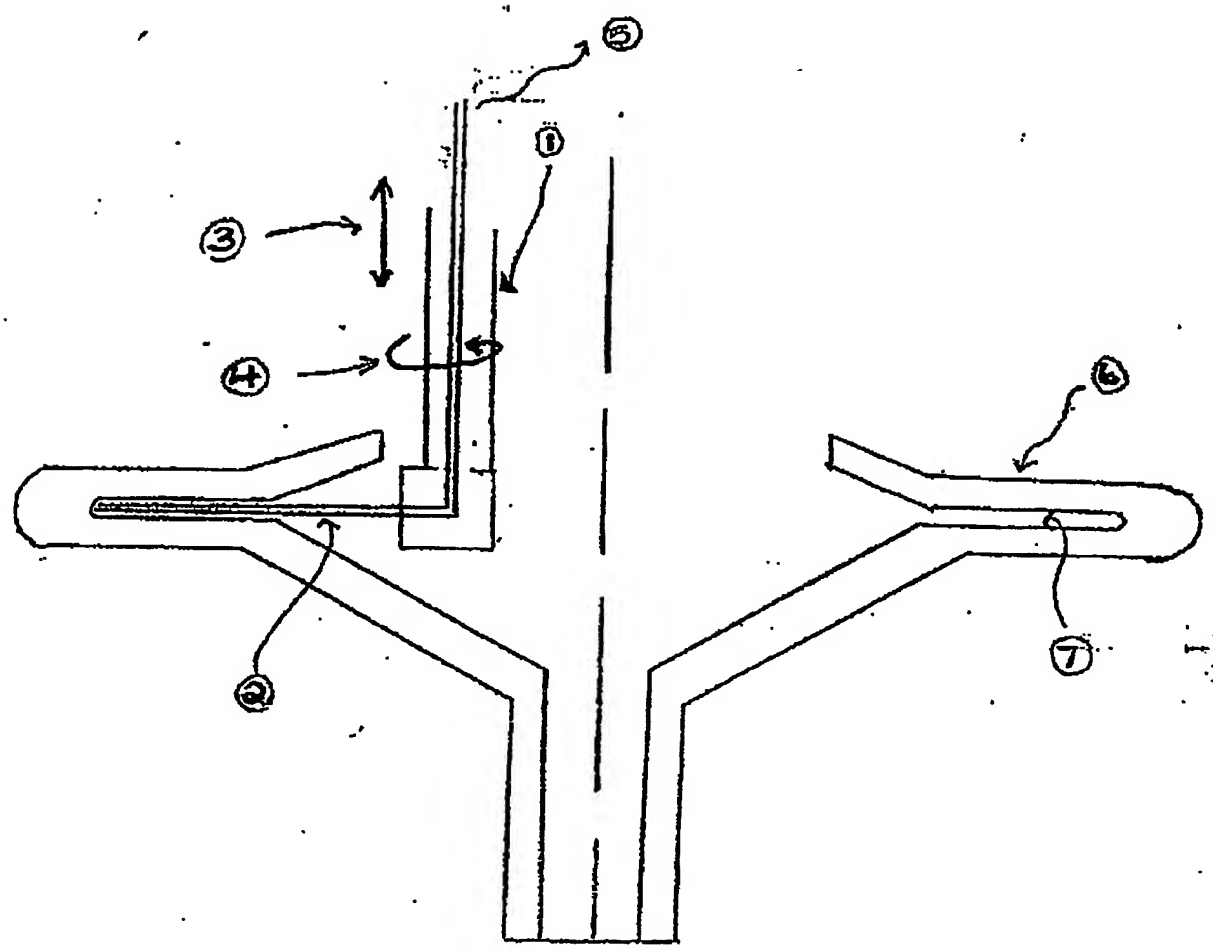


FIG. 9

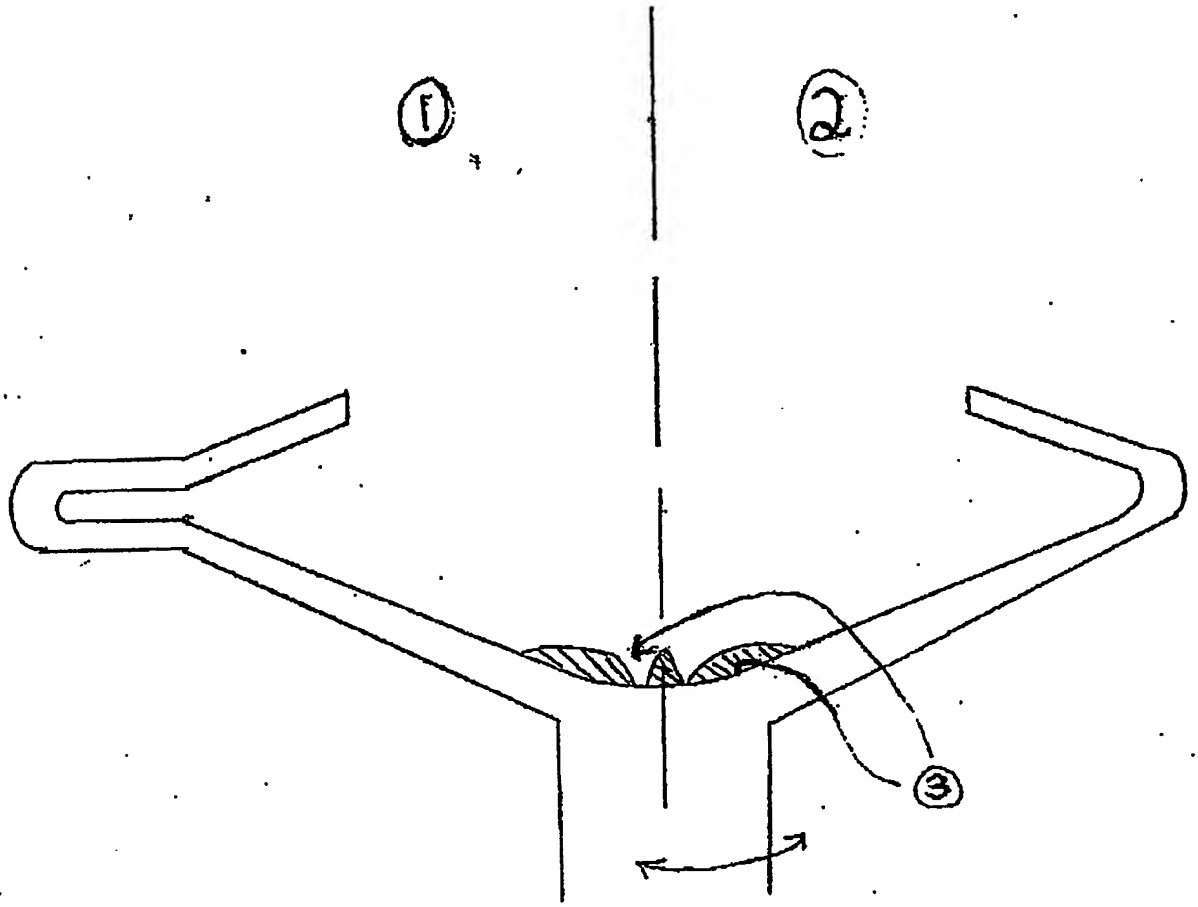


FIG. 10

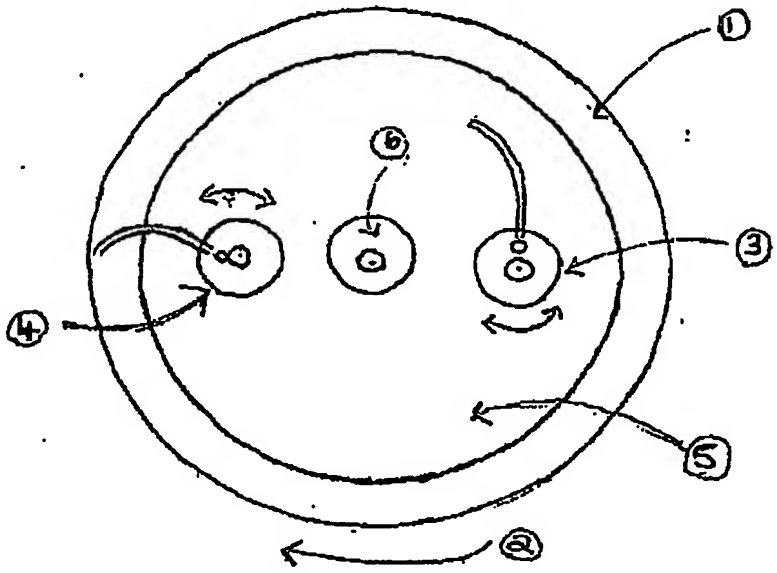


FIG. 11

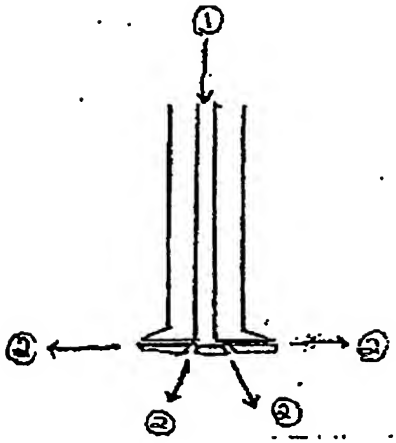


FIG. 11A

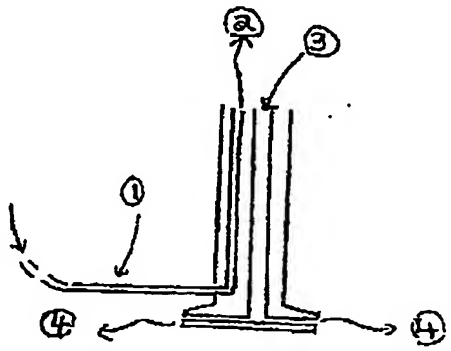


FIG. 11B

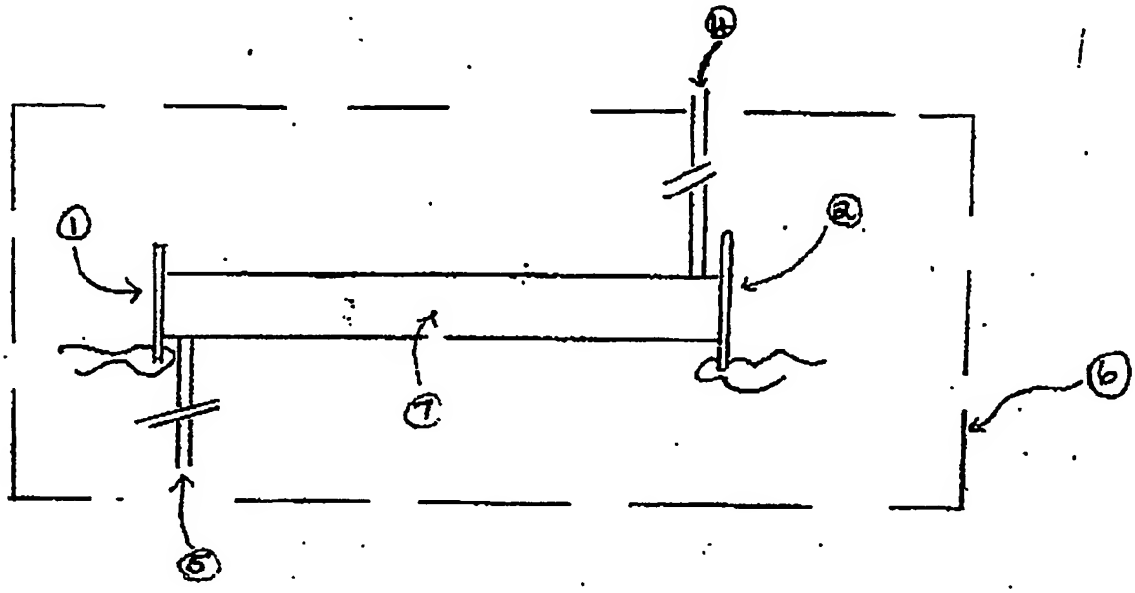


FIG. 12

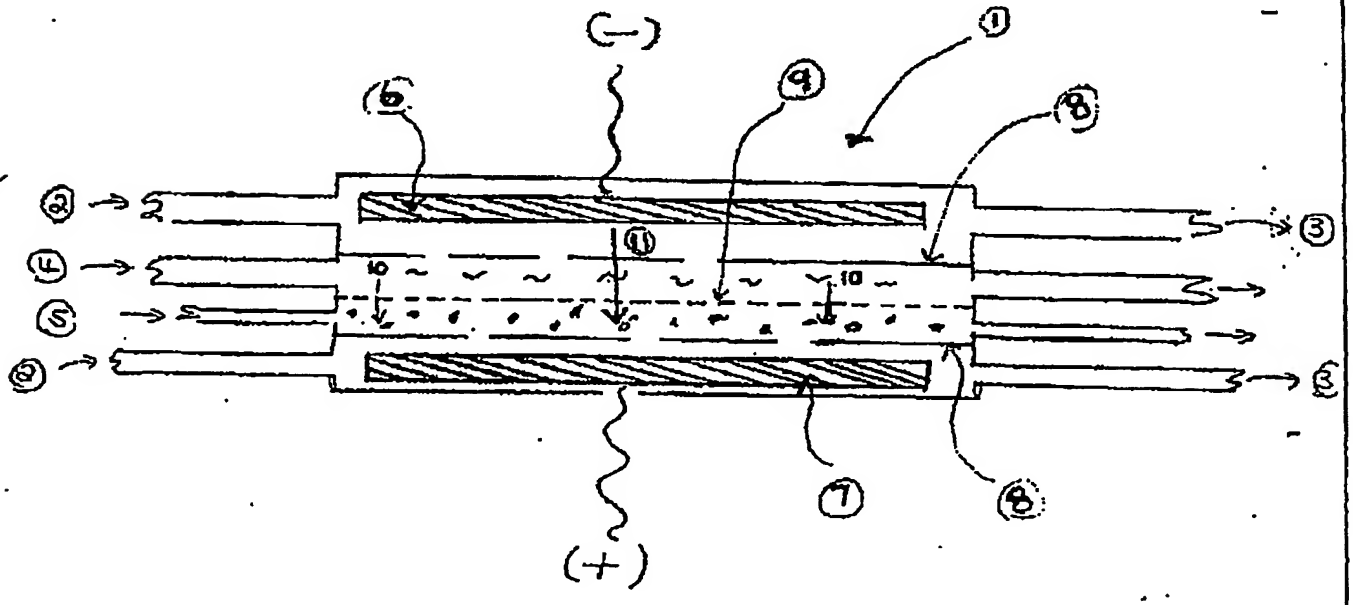


FIG. 13

FLOW CHART FOR AUTOMATED PLANT DNA PURIFICATION

Collect tissue samples into SAMPLER Receptacles

↓

Transport tissue samples to RoboPrep Automated Mill

↓

Homogenise the tissue through the RoboPrep Mill

↓

Mix tissue homogenate to release maximal yield of nuclei

↓

Phase-partion free Nuclei in Mixer-Flow Centrifuge 1 - low speed centrifugation

↓

Collect Nuclei-rich interface suspension

↓

Digest Nuclei using protease in Mixer-Flow Centrifuge 2

↓

Partition DNA into Lower Phase - low speed Centrifugation

↓

Collect DNA-rich Lower Phase

↓

Bind DNA onto Affinity Matrix and wash Matrix to remove residual digestion and phase buffers

↓

Elute DNA off Affinity Matrix into a small volume of Potassium Iodide solution

↓

Denature and Precipitate Genomic DNA with cold Alcohol

↓

Collect pure DNA onto Ultra-fine Membrane by Vacuum Filtration

↓

Wash pure DNA with Ethanolic buffer to remove residual salts, Vacuum dry

↓

Dissolve pure Genomic DNA in pure Water and distribute into 96-well Array

Figure 14

FLOW CHART FOR AUTOMATED PLANT DNA PURIFICATION

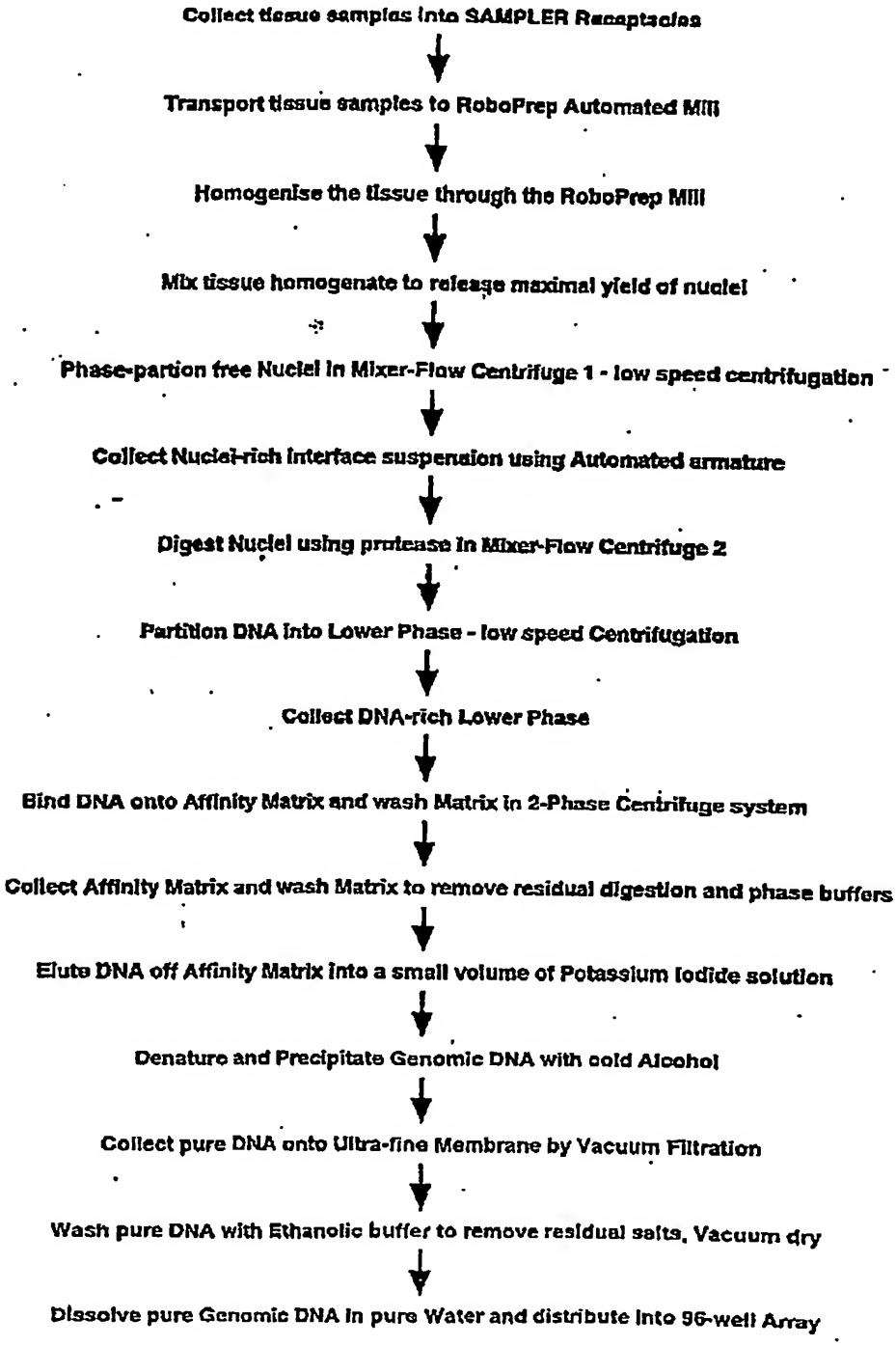


Figure 15

FLOW CHART FOR AUTOMATED PLANT DNA PURIFICATION

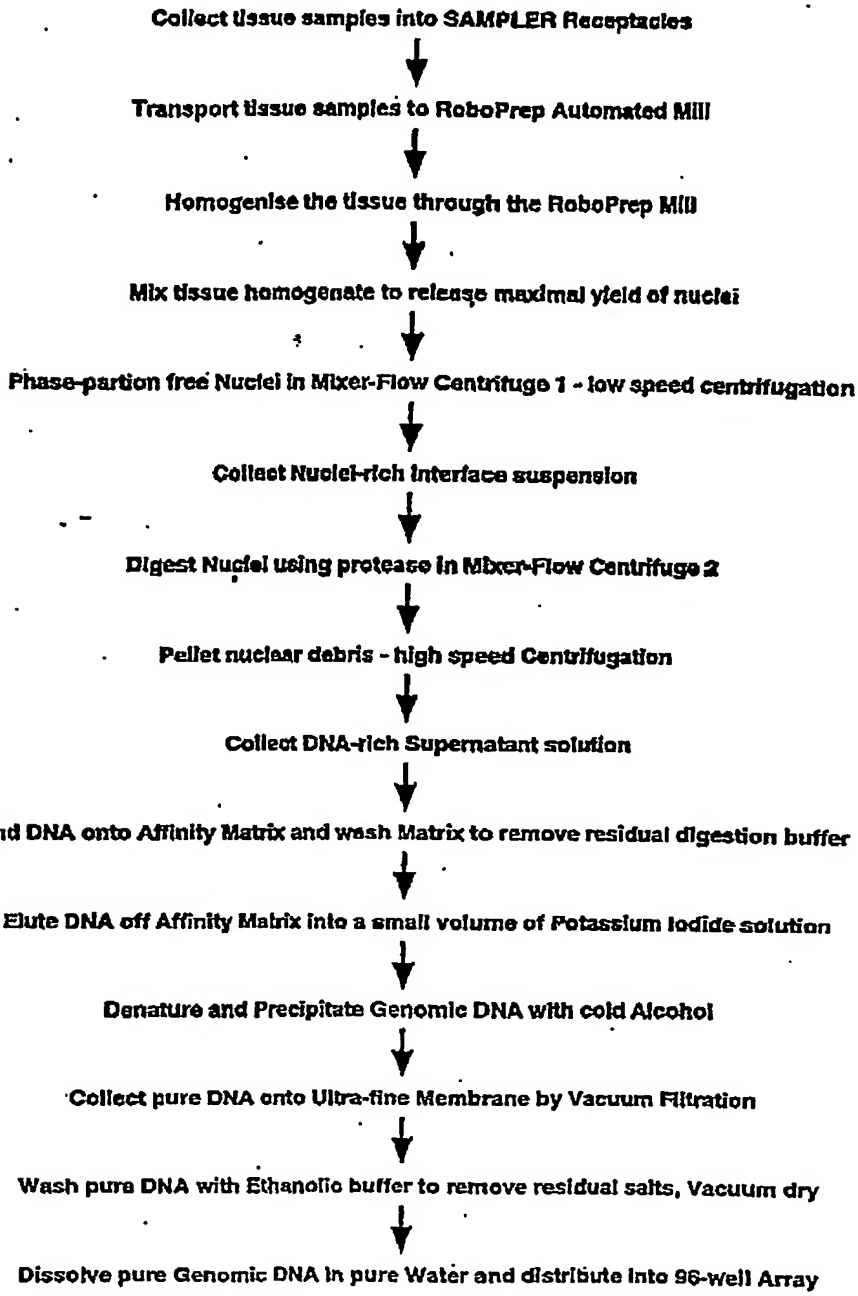


Figure 16

FLOW CHART FOR AUTOMATED PLANT DNA PURIFICATION

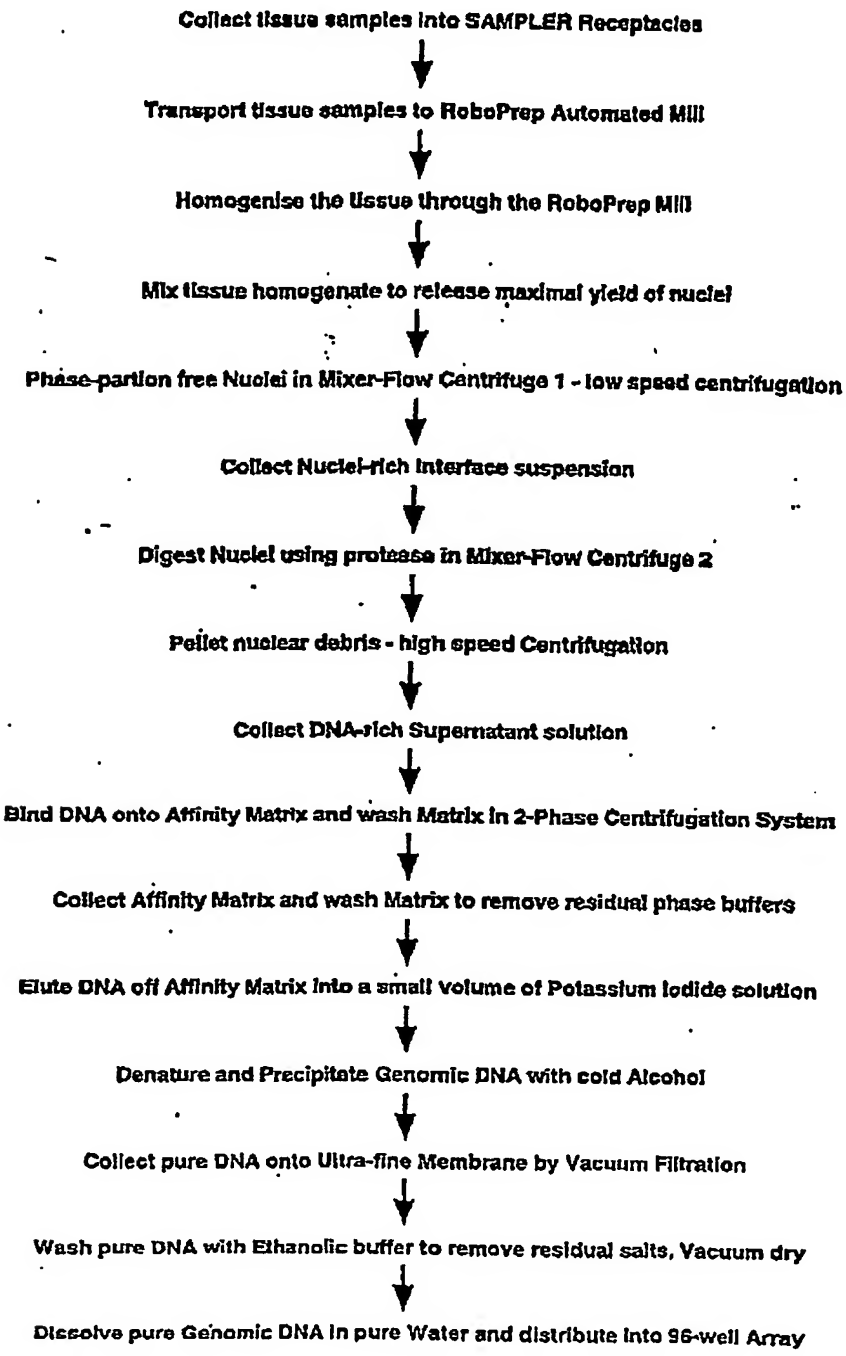


Figure 17

FLOW CHART FOR AUTOMATED PLANT DNA PURIFICATION

Collect tissue samples into SAMPLER Receptacles

↓

Transport tissue samples to RoboPrep Automated Mill

↓

Homogenise the tissue through the RoboPrep Mill

↓

Mix tissue homogenate to release maximal yield of nuclei

↓

Phase-partion free Nuclei in Mixer-Flow Centrifuge 1 at low speed centrifugation

↓

Collect Nuclei-rich interface suspension using Automated armature

↓

Digest Nuclei using protease in Mixer-Flow Centrifuge 2

↓

Partition DNA into Lower Phase - low speed Centrifugation

↓

Collect DNA-rich Lower Phase

↓

Electrophorese DNA onto Affinity Matrix within Electrophoresis Flow Cell

↓

Wash Affinity Matrix within Flow Cell to remove residual digestion and electrophoresis buffers

↓

Elute DNA off Affinity Matrix into a small volume of Potassium Iodide solution

↓

Denature and Precipitate Genomic DNA with cold Alcohol

↓

Collect pure DNA onto Ultra-fine Membrane by Vacuum Filtration

↓

Wash pure DNA with Ethanolic buffer to remove residual salts, Vacuum dry

↓

Dissolve pure Genomic DNA in pure Water and distribute into 96-well Array

Figure 18

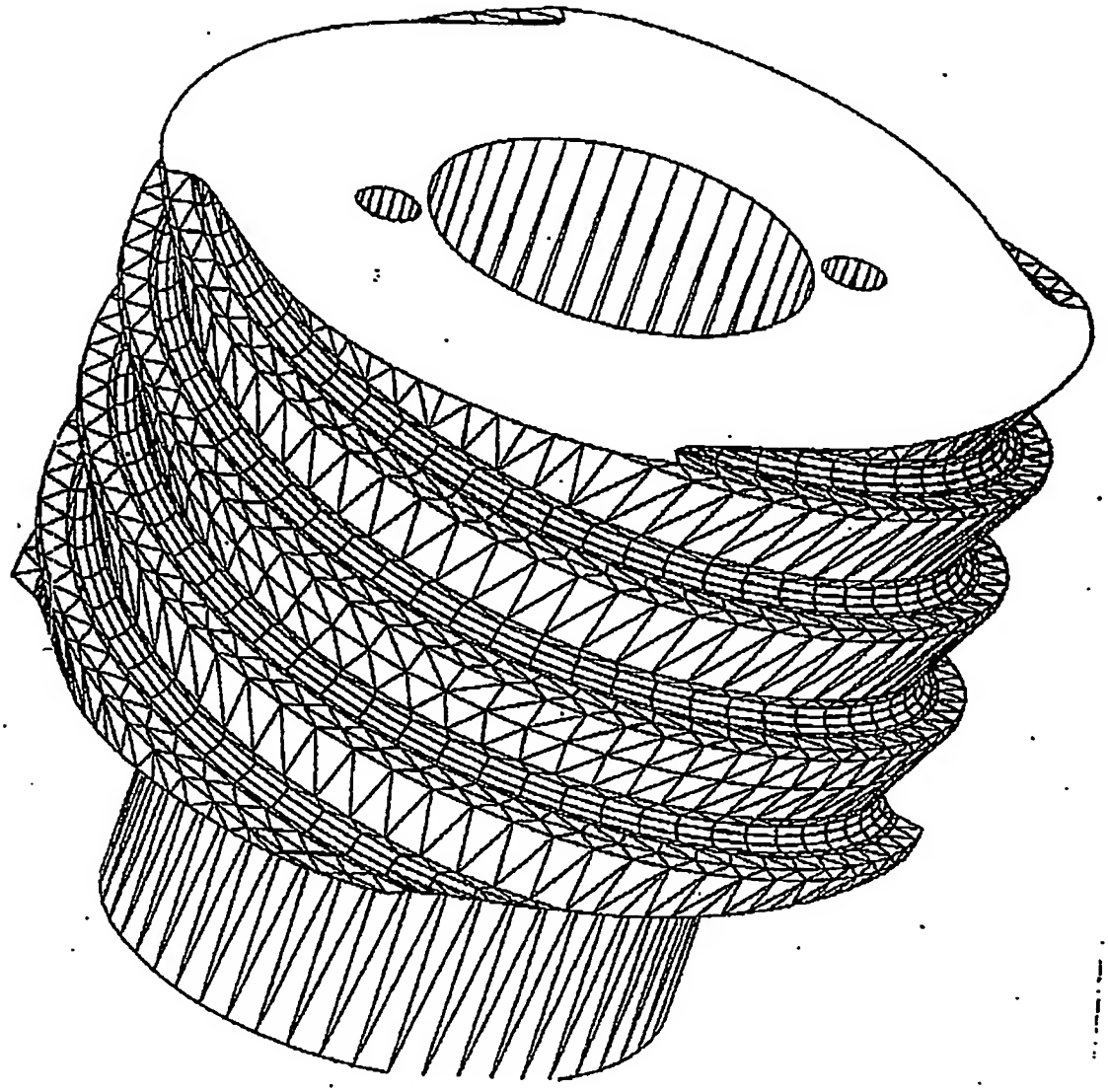


FIG. 19

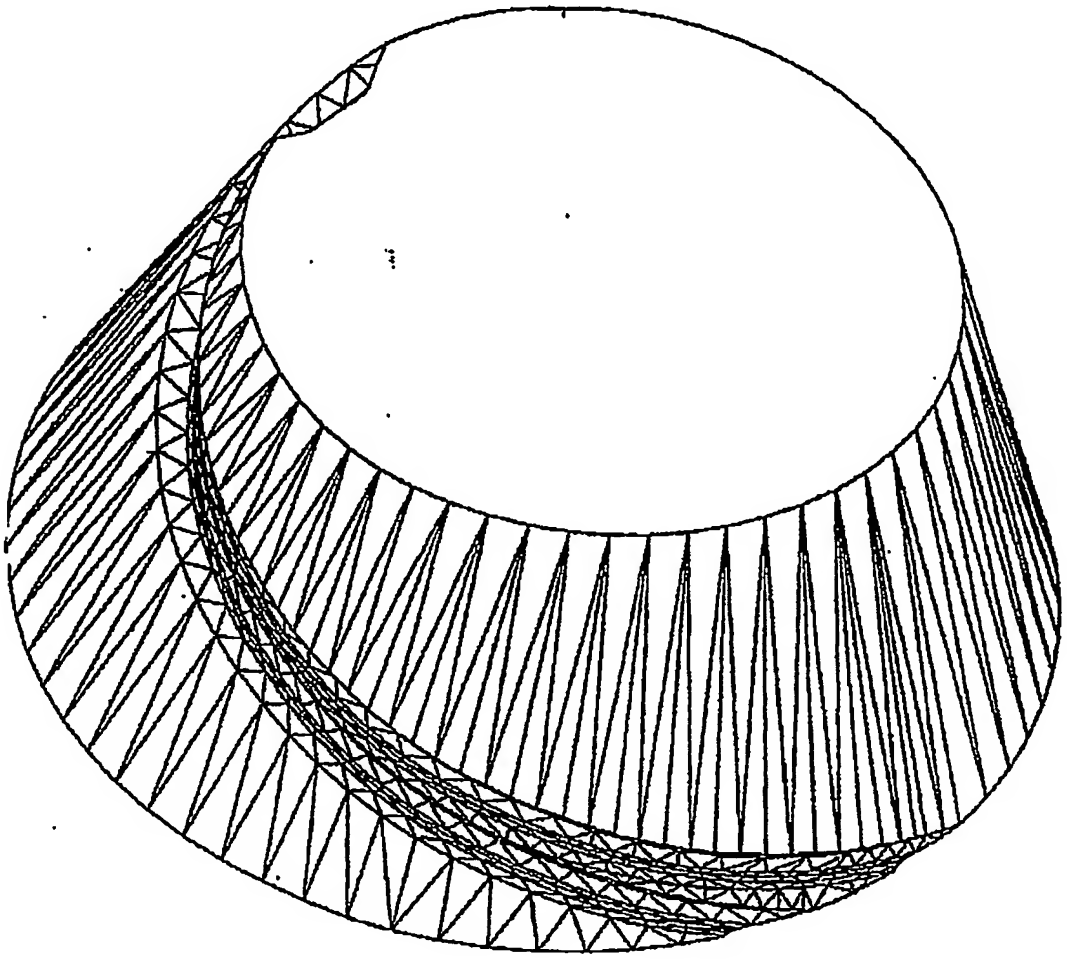


FIG. 20

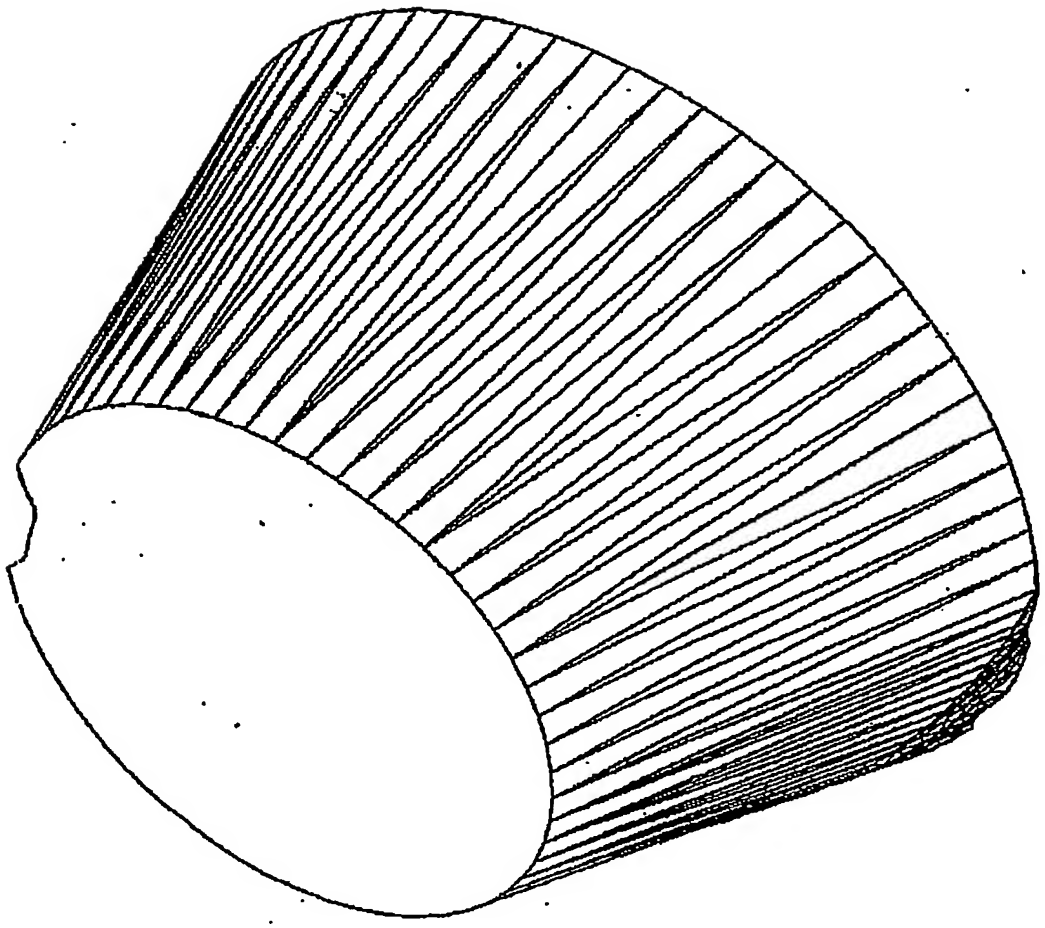


FIG. 21

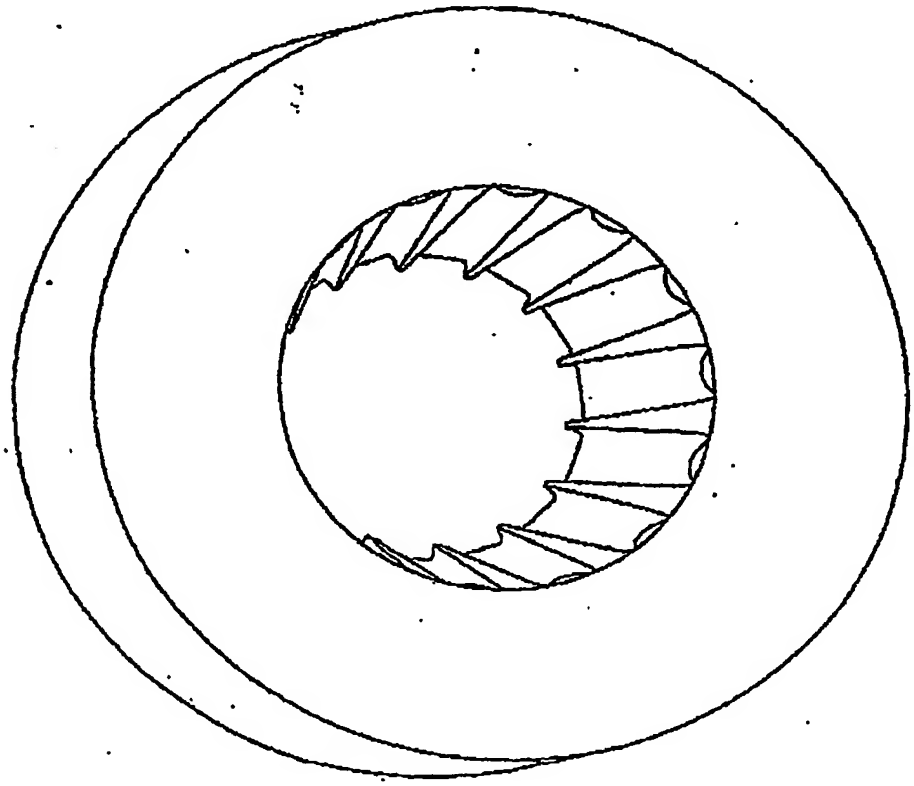


FIG. 22

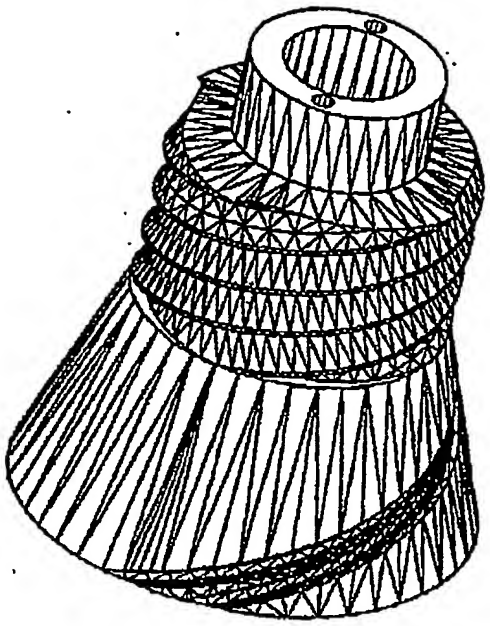


FIG. 23

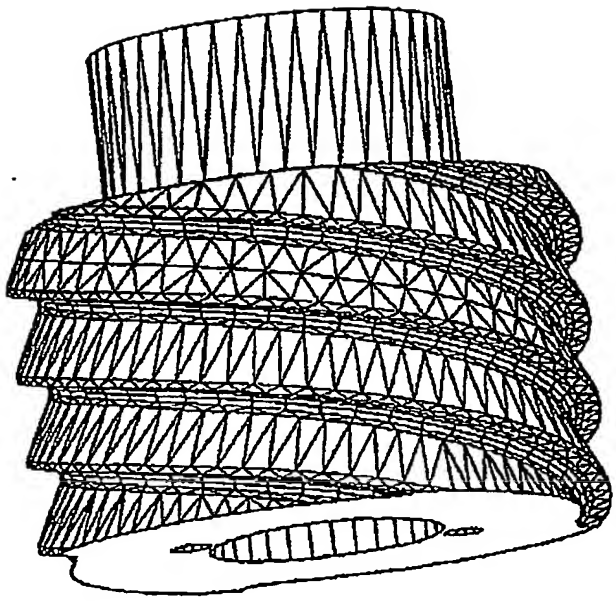


FIG. 24

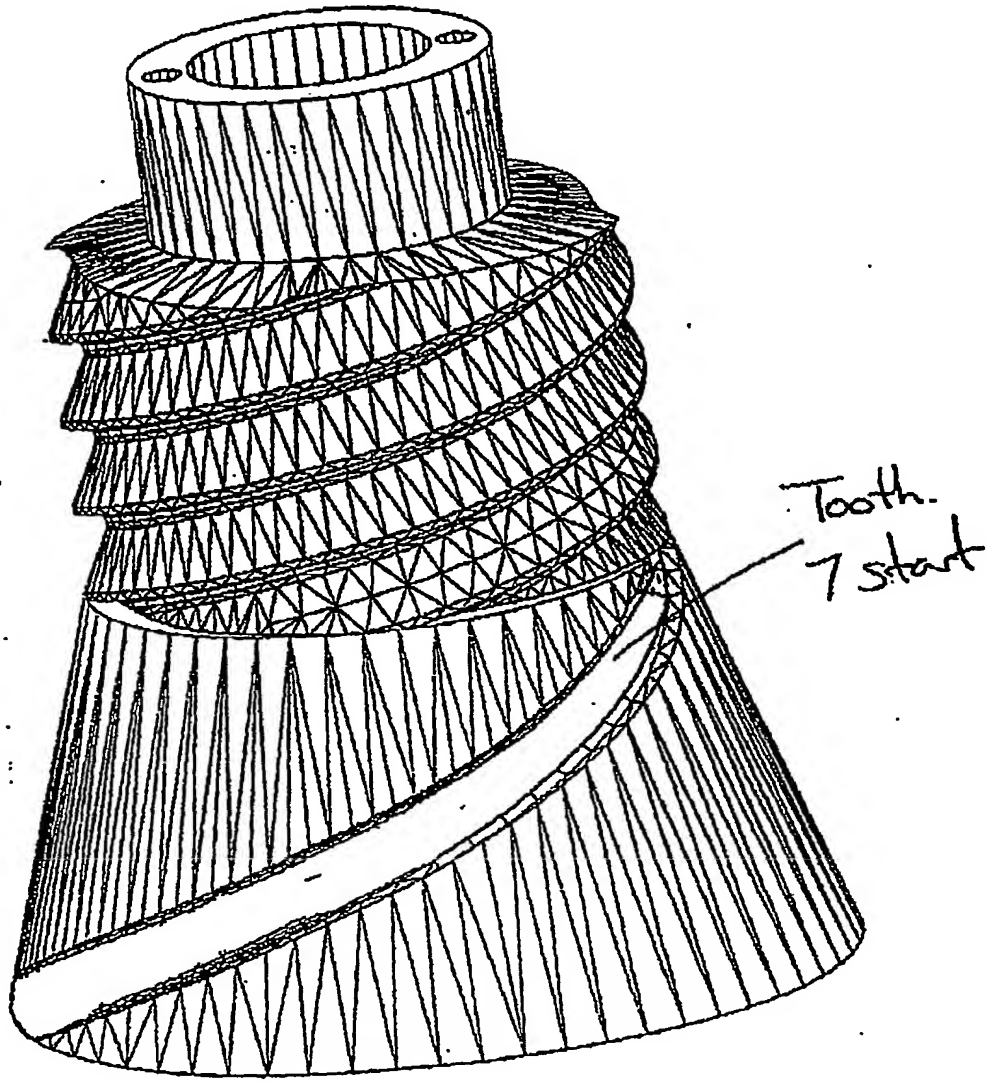


FIG. 25